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Award Number: W81XWH-04-1-0819

TITLE: The Role of TSC Proteins in Regulating Cell Adhesion and Motility

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REPORT DATE: September 2006

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01-09-2006	Final	23 Aug 2004 – 22 Aug 2006

4. TITLE AND SUBTITLE

The Role of TSC Proteins in Regulating Cell Adhesion and Motility

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-04-1-0819

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Pennsylvania
Philadelphia, Pennsylvania 19104-6270

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The goal of the proposal was to define the molecular signaling mechanisms by which TSC1 and TSC2 proteins regulate cell adhesion and motility as it relates to the genetic disorder tuberous sclerosis complex (TSC). The central hypothesis of the proposal was: TSC proteins regulate cell adhesion and motility, and loss of either TSC1 or TSC2 function alters cell adhesion and induces aberrant motility promoting the pathological conditions associated with TSC. We examined the function of TSC1 and TSC2 in regulating cell dynamics, and found that loss of TSC2 results in TSC1-induced activation of RhoA and Rac1 inhibition, which induces the increased migration and invasiveness of primary human smooth muscle-like LAM-derived (LAMD) cells, derived from tumors, and TSC2-/- MEFs. Re-expression of TSC2 reverses these effects. Depletion of TSC2 with specific siRNA in NIH 3T3 fibroblasts also results in increased migration. Increased LAM cell migration is rapamycin-insensitive, suggesting a mTORC1-independent mechanism. Our data indicate that TSC2 not only functions as a negative regulator of cell growth, but also acts as a negative regulator of cell migration; thus, loss of TSC2 not only results in abnormal cell growth but also dysregulates cell motility, which leads to a metastatic phenotype.

15. SUBJECT TERMS
No subject terms provided.

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	49	**19b. TELEPHONE NUMBER** *(include area code)*

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

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Introduction:

The goal of this project was to define the molecular signaling mechanisms by which TSC1 and TSC2 proteins regulate cell adhesion and motility as it relates to the genetic disorder tuberous sclerosis complex (TSC). The pathogenesis of TSC that develops due to the loss-of-function of tumor suppressors TSC1 and TSC2 proteins represents an extremely complex, and not fully understood, interplay of deregulated cell functions. The neurological manifestations of TSC are related to brain lesions named tubers that have been defined as a 'neuronal migration disorder' and occur due to aberrant neuronal motility during brain development. As a result of aberrant neuronal motility, affected individuals may suffer from seizures, mental retardation, and autism. Thus, TSC represents a major cause of developmental disorders and epilepsy in the pediatric population.

The central hypothesis of this proposal was: TSC proteins regulate cell adhesion and motility, and loss of either TSC1 or TSC2 function alters cell adhesion and induces aberrant motility promoting the pathological conditions associated with TSC. PI3K and small GTPases RhoA and Rac1, respectively, serve as the upstream modulator and downstream effectors of TSC1 and TSC2 proteins.

The significance of this work relates to better understanding of the molecular and cellular mechanisms of the pathobiology of TSC such that new therapeutic targets can be identified to treat this devastating disease.

Body:

Task 1. Determine whether loss or mutation of TSC1 and TSC2 proteins modulate cell adhesion and motility

During the two-year period of this grant, substantial advance in our understanding of the signaling molecular mechanisms o TSC1/TSC2-dependent regulation of cell dynamics was achieved.

Abnormal cell migration and invasiveness are among the characteristic features of tumors, which may occur, in part, due to the deregulation of TSC2 signaling, and could be augmented by promigratory growth factors, such as PDGF and serum containing the critical promigratory factor lysophosphatidic acid (LPA).

We have recently published paper (**Modulation of cell migration and invasiveness**

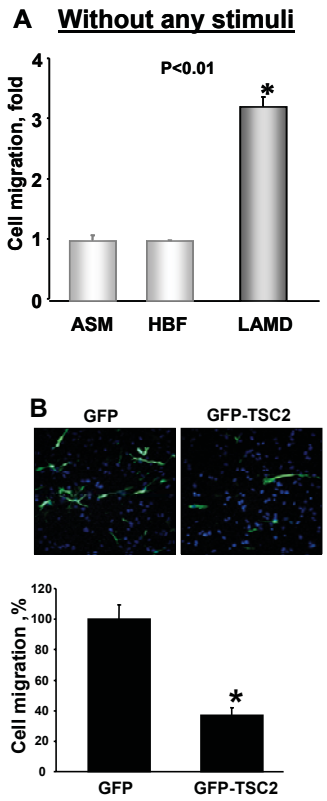


Figure 1. Increased migration of LAMD cells is inhibited by TSC2. A: Serum-deprived LAMD, airway smooth muscle (ASM) cells and human bronchus fibroblasts (HBF) were placed on collagen-saturated membranes in serum-free medium, and allowed to migrate in the Boyden chamber for 4 h in the absence of any stimuli then membranes were fixed, stained with Hemacolor stain set, and analyzed using Gel Pro software. Data represent mean values ± SE from measurements performed in triplicate from three separate LAMD cell lines by ANOVA (Bonferroni-Dunn test). B: TSC2 expression inhibits LAMD cell migration. Cells were transiently transfected with plasmids expressing GFP-tagged TSC2 and control GFP. Then cell transfection efficiency was assessed by visual quantitative analysis, followed by migration assay; membranes were fixed and then immunostained with anti-GFP antibody to detect GFP-TSC2 and GFP. Data represent the percentage of migrated cells per number of cells transfected with either GFP- or GPF-TSC2-expressing plasmids, and presented as mean values ± SE from three observations. *P < 0.001 for cells transfected with GFP-TSC2 or TSC2-N vs. cells transfected with GFP by ANOVA (Bonferroni-Dunn test)[1].

by tumor suppressor TSC2 in LAM. Goncharova, E. A., Goncharov, D. A., Lim, P. N., Noonan, D. & **Krymskaya, V. P.** American Journal of Respiratory Cell and Molecular Biology, v.34: 473-480, 2006, See Appendix) demonstrating that primary cultures of human TSC2-deficient LAM-derived (LAMD) cells exhibit an increased migratory activity and invasiveness (Figs 1A and 2A), which is abolished by TSC2 re-expression (Figs 1B and 2B).

We found that the small GTPase RhoA is constitutively activated in the absence of any stimuli in serum-deprived LAMD cells (Fig. 3A). In contrast, HBFs showed a low level of basal RhoA activity in serum-deprived LAMD cells (Fig. 3A). Importantly, expression of TSC2 significantly inhibited RhoA activity compared to cells transfected with control plasmid (Fig. 3B). Importantly, pharmacological inhibition of RhoA activity abrogates LAMD cell migration (Fig. 4), suggesting that Rho modulates LAMD cell migration. Thus, these data demonstrate that TSC2 play a critical role in modulating cell migration and invasiveness.

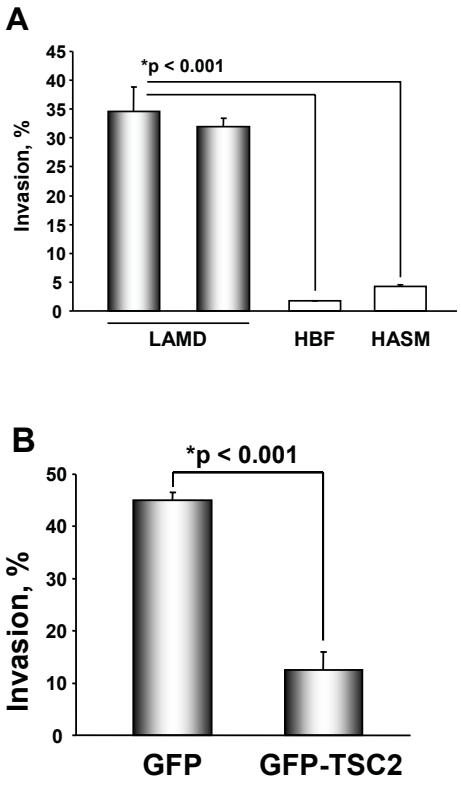


Figure 2. A: LAMD cells have increased invasiveness[1]. Invasiveness of LAMD cells was analyzed using the Cultrex 96 Well BME Cell Invasion Assay kit according to manufacture's protocol. Briefly, serum-deprived cells from two different LAMD cell lines, HASM cells, and HBFs were placed in the upper wells of the Cultrex cell invasion chamber, pre-coated with BME, and incubated for 24 h in a CO_2 incubator. After incubation non-invaded cells were washed away; invaded cells were incubated with Cell Dissociation/Calcein AM Solution, and fluorescence was measured using a Fluorescent 96-well plate reader (485 nm excitation, 520 nm emission). The quantity of invaded cells was calculated using standard curves, separate for each cell type. Data represent % of invaded cells per total number of cells was taken as 100%. B: TSC2 expression inhibits LAMD cell invasiveness. Cells were transfected with GFP-TSC2 or GFP as a control followed by the cell invasion assay. Data represent mean values ± SE from two independent experiments by ANOVA (Bonferroni-Dunn test).

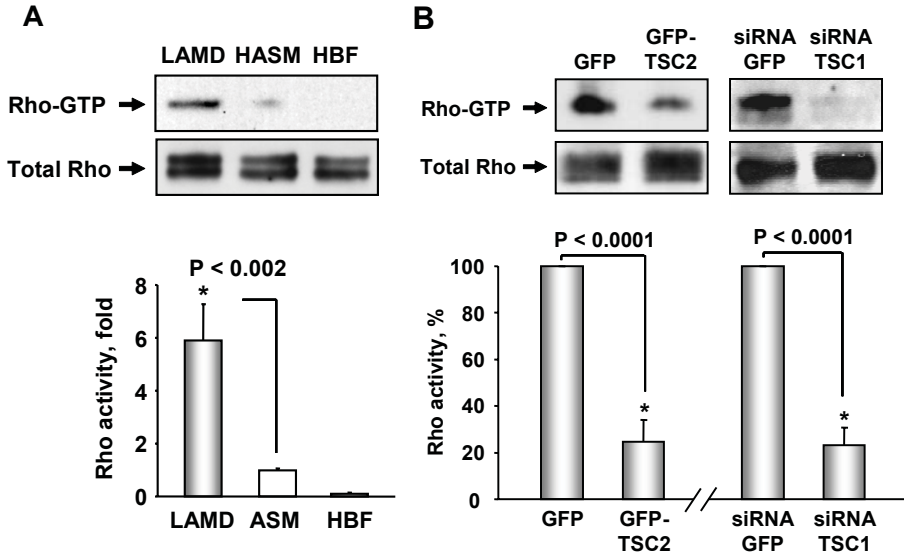


Figure 3. Increased Rho activity in LAMD cells is inhibited by TSC2 expression or TSC1 depletion. A: activated GTP-bound RhoA was pulled-down with RBD agarose from serum-deprived LAMD, ASM cells and HBFs cell lysates, followed by immunoblot analysis with anti-RhoA antibodies. B: LAMD cells were transfected with either GFP-TSC2 and control GFP, or siRNA TSC1 and control siRNA siGLO RISC-Free, followed by the Rho activity assay[1].

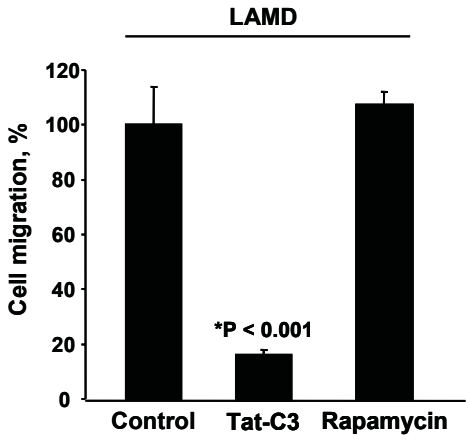


Figure 4. RhoA modulates LAMD cell migration. Serum-deprived cells were treated with either 0.5 μM Tat-C3 or 200 nM rapamycin; next the migration assay was performed using a Boyden chamber. Data are representative of 6 replicates for each condition in 2 independent experiments. *P < 0.001 for Tat-C3-treated cells vs. control by ANOVA (Bonferroni-Dunn test).

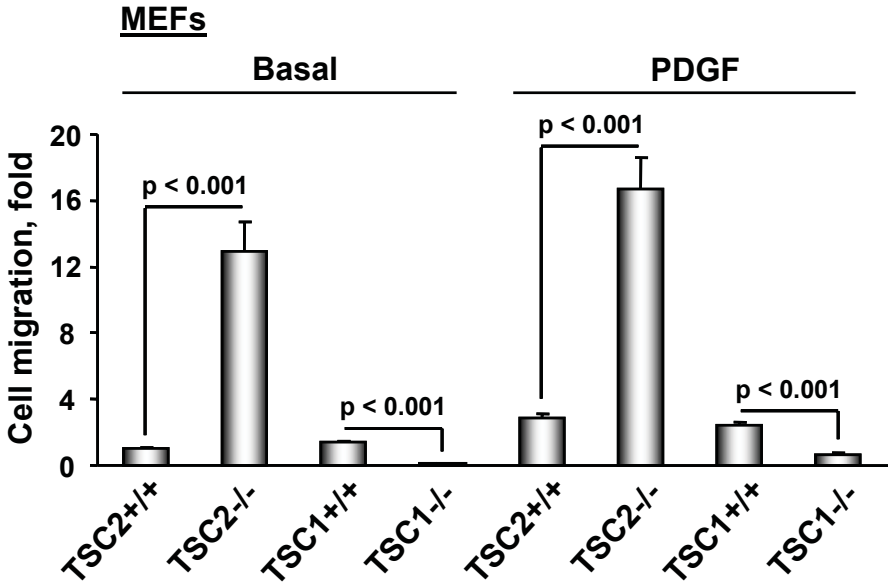


Figure 5. Increased migration of TSC2-/- MEFs is inhibited by TSC2 re-expression. Serum-deprived or 10 ng/ml PDGF-stimulated TSC2+/+, TSC2-/-, TSC1+/+, and TSC1-/- MEFs were placed on collagen-saturated membranes in serum-free medium, and allowed to migrate in the Boyden chamber for 4 h in the absence of any stimuli then membranes were fixed, stained with Hemacolor stain set, and analyzed using Gel Pro software. Data represent mean values ± SE from measurements performed in triplicate from three separate LAMD cell lines by ANOVA (Bonferroni-Dunn test). Each experimental condition is represented in triplicate. C: Statistical analysis of migration experiments. Data represent mean values ± SE from six repetitions by ANOVA (Bonferroni-Dunn). Basal migration of TSC2+/+ cells was taken as 1 fold.

To investigate whether TSC1 is also required for TSC2-dependent RhoA inhibition, we used siRNA directed against TSC1 and down-regulated TSC1 levels.

As seen in Figure 3B, the down-regulation of TSC1 levels resulted in the significant inhibition of constitutive RhoA activation in LAMD cells. Because TSC2 inhibits RhoA activity and TSC1 siRNA is sufficient for this inhibition, we conclude that TSC1 is required for the TSC2-dependent regulation of RhoA activity. This data show that TSC2 acts upstream of RhoA, and suggest that TSC2-dependent inhibition of LAMD cell migration occurs due to the TSC2-dependent inhibition of RhoA activity. These data also demonstrate that RhoA is activated in LAMD cells, and suggest that RhoA activation may contribute to invasive LAM cell migration.

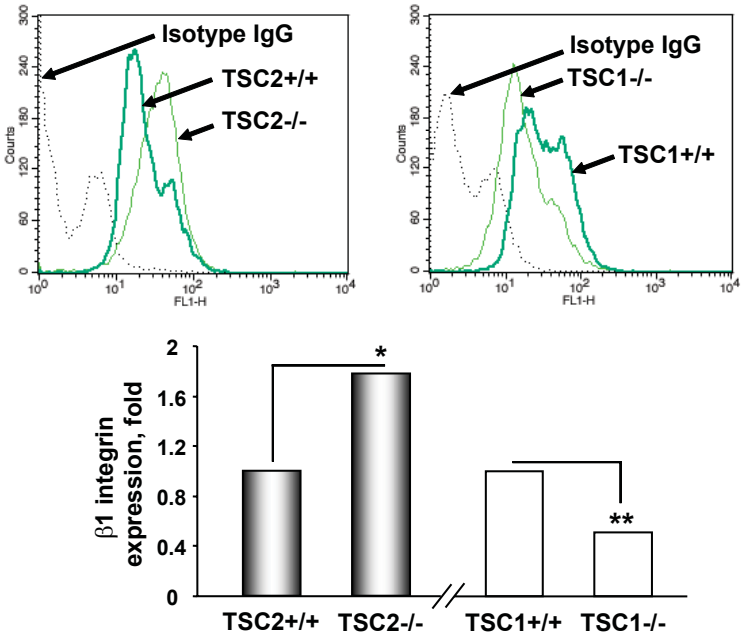


Figure 6. Differential expression of integrins in TSC1-/- and TSC2-/- MEFs. FACS analysis of β1 integrin expression in TSC1-/-, TSC2-/-, TSC1+/+, and TSC2+/+ MEFs using primary rat anti-mouse monoclonal and FITC -conjugated secondary antibody (BD Biosciences, Cat #553715), and isotype-matched IgG as a negative control. The median fluorescence intensity (MFI) for β1 integrin in wild type TSC2+/+ and TSC1+/+ cells, respectively, was taken as 1 fold.

To confirm our findings obtained in the primary human LAMD cells, we examined the migratory activity of established cell cultures of TSC2-/- MEFs. Migration of serum-deprived TSC2-/- cells, without any stimuli, was markedly increased compared to wild type TSC2+/+ MEFs (Fig 5). Interestingly, TSC1-/- MEFs migrated markedly slower compared to wild type TSC1+/+ MEFs; these changes were not due to the decreased survival of TSC1-/- MEFs (data not shown). As shown in Figure 6, the differences in migration rates are potentially associated with TSC1-dependent modulation of cell adhesion due to the decreased expression of β1 integrin. Collectively, these data indicate that loss of TSC2 increases cell migration, thus suggesting that TSC2 function as a negative regulator of cell motility, and loss of its function may result in the abnormal cell migration.

To explore whether TSC2 regulates cell motility, we performed live imaging of the

wound closure of TSC2-deficient smooth muscle ELT3 cells, which were either untreated or transduced with a replication deficient adenovirus expressing GFP-tagged wild type TSC2 or control GFP (Fig. 7). TSC2-/- cells retained their motile properties; however their movement was characterized by the formation of short lamellipodia at the leading edge of migrating cells. Expression of GFP-tagged TSC2 markedly changed the pattern of cell dynamics; during wound closure moving cells formed dynamic membrane protrusions, and the rate of membrane extension for GFP-TSC2-infected cells was 1.00 ± 0.25 μm x min^{-1} compared to 0.35 ± 0.08 μm x min^{-1} for GFP-infected cells. Expression of control GFP in TSC2-/- cells had little effect on the pattern of cell movement. These data demonstrate that re-expression of TSC2 markedly changes TSC2-/- cell morphology during cell motility, and suggest that TSC2 might be important for the formation of membrane protrusions during directional movement. These data were published in our paper untitled "**TSC2 modulates actin cytoskeleton and cell adhesion through TSC1-binding domain and Rac1 GTPase"** Goncharova E.A., Goncharov D.A., Noonan D., **V.P. Krymskaya**. Journal of Cell Biology. 167: 1171-1182, 2004.

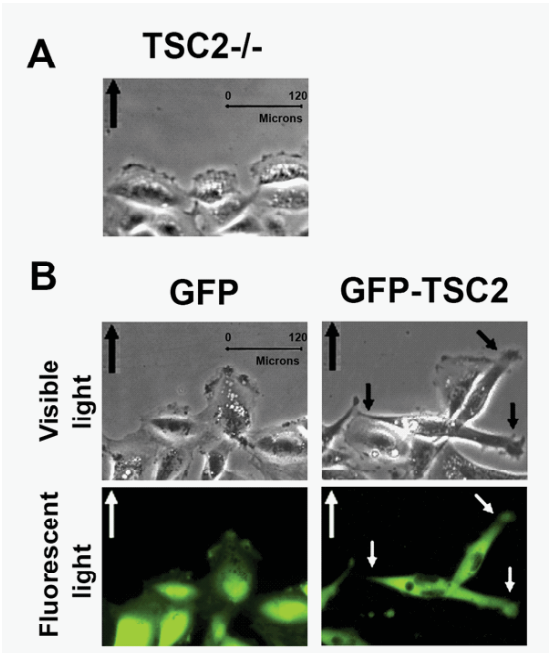


Figure 7. Representative images of live cell image analysis demonstrating that re-expression of TSC2 modulates TSC2-/- cell morphology and the dynamics during wound closure [2].

Because cell motility requires focal adhesion remodeling, we examined whether TSC2-induced changes in motility correlates with changes in focal adhesion formation. Immunohistochemical analysis with anti-vinculin antibody showed that in TSC2-/- cells focal adhesions were localized throughout the cell as well as at the cell periphery . Expression of TSC2 promoted marked changes in the shape and size of the focal adhesions: most focal adhesions in the center of the cells were disassembled, and the quantity of focal adhesions per cell was attenuated by 30.3 ± 5.9 % compared to control. At the same time the size of focal adhesions at the cell periphery was also markedly decreased. Importantly, TSC2-HBD, which is involved in TSC1/TSC2 complex formation, also promoted focal adhesion disassembly in the center of the cells. TSC1 regulates cell adhesion and its inactivation results in the loss of focal adhesions. To determine whether focal adhesion remodeling in TSC2-/- cells requires TSC1, we microinjected siRNA TSC1, and found that down-regulation of TSC1 promoted focal adhesion disassembly in TSC2-/- cells. These results demonstrate that expression of the TSC1 binding domain of TSC2 (TSC2-HBD) and the down-regulation of TSC1 with siRNA TSC1 are sufficient for focal adhesion disassembly, which suggest that TSC2 involves TSC1 in regulating focal adhesion remodeling [2].

Because the cortical actin staining observed in TSC2-transfected cells was reminiscent of the effects of small GTPase Rac1, and since Rac1 induces the formation of membrane ruffles and lamellipodia, which ultimately results in focal adhesion remodeling we examined the effect of the constitutively active form of Rac1 (V12Rac1) on focal adhesion formation in TSC2-/- cells. Vinculin immunostaining showed that V12Rac1 expression induced marked changes in focal adhesion formation similar to effects of TSC2, suggesting a potential link between TSC2 and Rac1 signaling.

Task 2. Determine whether Rho GTPase is a downstream effector of TSC1- and TSC2-dependent cell adhesion and motility

According to the existing model describing role of Rho GTPases in actin remodeling in normal

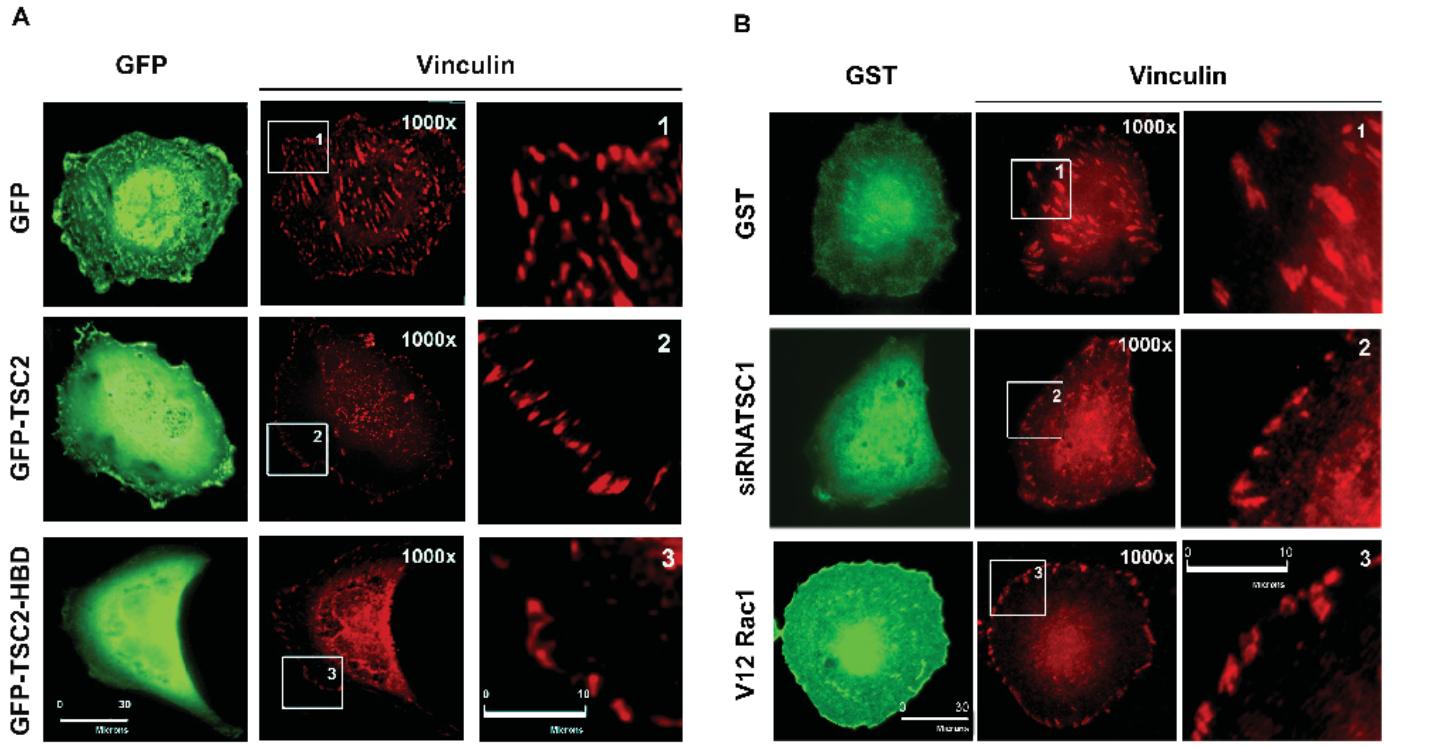


Figure 8. TSC2, TSC2-HBD and siRNA TSC1 modulate focal adhesion formation. A: Cells were transfected with control GFP (top panel), GFP-TSC2 (middle panel), or GFP-TSC2-HBD (bottom panel) plasmids, and then immunostained with anti-GFP (green) and anti-vinculin (red) antibodies to detect focal adhesions. Representative images of three separate experiments were taken on a Nikon Eclipse TE2000-E Microscope at 1000X magnification. B: Cells were transfected with control GST, GST-tagged V12Rac1, or siRNA TSC1 co-microinjected with GST to identify microinjected cells, followed by immunostaining with anti-GST (green) and anti-vinculin (red) antibodies. Representative images are from 138 microinjected cells [2].

untransformed cells, Rac1 activation promotes membrane protrusions at the leading edge, and RhoA activation regulates contractility in the cell body. Growing evidence, especially in cancer studies, suggest that either RhoA or Rac1 activation contribute to increased cell migration depending on the cell context or the cell type. Additionally, RhoA activity is often elevated in tumors and activated ROCK induces tumor dissemination in vivo. Recent data uncover paradigm-shifting evidence about role of Rho activation in promoting cell migration. Thus, using biosensor with intramolecular fluorescence resonance energy transfer (FRET), increased RhoA activity was found to be concentrated in a sharp band directly at the edge of membrane protrusions in migrating cells.

We hypothesized that in quiescent cells, TSC1 and TSC2 exist in the form of membrane complex and the binding of TSC2 through TSC2-HBD with TSC1 inhibits TSC1-dependent Rho activation. Upon growth factor stimulation or due to TSC2 mutations, TSC2 dissociates from or does not form a complex with

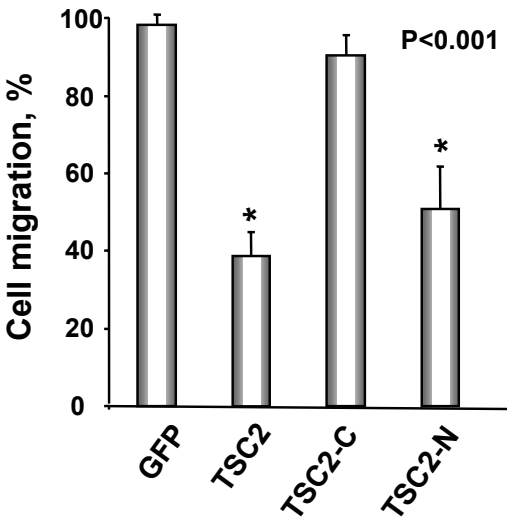


Figure 9. N-terminal of TSC2 is sufficient to inhibit LAMD cell migration. Cells were transiently transfected with plasmids expressing GFP-tagged TSC2, N-terminal (TSC2-N), C-terminal (TSC2-C) of TSC2, and control GFP. Then cell transfection efficiency was assessed by visual quantitative analysis, followed by migration assay; membranes were fixed and then immunostained with anti-GFP antibody to detect GFP-TSC2 and GFP. Data represent the percentage of migrated cells per number of cells transfected with either GFP- or GPF-TSC2-expressing plasmids, and presented as mean values ± SE from three observations. *P < 0.001 for cells transfected with GFP-TSC2 or TSC2-N vs. cells transfected with GFP by ANOVA (Bonferroni-Dunn test)[1].

TSC1 thus releasing the Rho activating domain of TSC1 from negative regulation; this leads to Rho activation and increased cell migration. To test our hypothesis we examined whether the N-terminus of TSC2, containing TSC2-HBD, will be sufficient to inhibit increased migration of LAMD cells. As seen in Figure 9, most of the LAMD cells transfected with GFP (99.7±9.2%) show increased motility in the absence of any stimuli. In contrast, only 37.1±5.1% of the total number of cells expressing GFP-tagged TSC2 migrated into the collagen-saturated matrix in serum-free medium. We found that the N-terminal of TSC2 was sufficient, and as effective, in inhibiting cell migration as full-length TSC2.

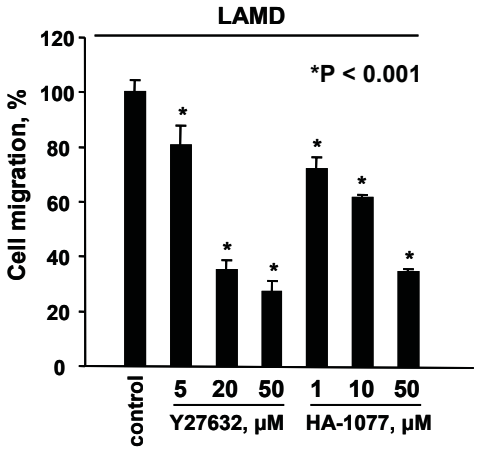


Figure 10. RhoA signaling modulates LAMD cell migration. Serum-deprived cells were treated with either Y27632 or HA-1077, followed by migration assay. Data are representative of 6 replicates for each condition in 2 independent experiments. *P < 0.001 for Y27632- or HA-1077-treated cells vs. control by ANOVA (Bonferroni-Dunn test)[1].

To determine whether RhoA activation modulates LAMD cell migration, we used Tat-C3 toxin, a cell-penetrating form of Clostridium botulinum exoenzyme, which catalyses the specific inactivation of RhoA. As seen in Figure 4, Rho inhibition markedly attenuated LAMD cell migration; in contrast, rapamycin (200 nM), a specific inhibitor of the mTORC1, had little effect on LAMD cell migration. Importantly, this concentration of rapamycin is sufficient to abrogate LAMD cell proliferation. To further examine the involvement of RhoA in LAMD cell migration, we treated LAMD cells with ROCK inhibitors Y27632 and HA-1077 at the range of concentrations which inhibit ROCK activity in other cell types. As seen in Figure 10, Y27632 and HA-1077 significantly inhibited LAMD cell migration in a concentration-dependent manner. In contrast, the same concentrations of Y27632 and HA-1077 had little effect on the migration of HBFs (data not shown). These data demonstrate that RhoA and ROCK activation is critical for LAMD cell migration.

To clarify the function of small GTPase Rac1 in TSC2-induced focal adhesion remodeling we examined Rac1 activation in TSC2-/- cells. By expressing TSC2 in TSC2-/- cells, we found that TSC2 alone was sufficient to markedly increase Rac1 activity compared to cells transfected with control GFP. Similarly, Rac1 activity was increased in LAMD cells transfected with TSC2, indicating that TSC2 may elicit activation of Rac1. Importantly, down-regulation of TSC1 with TSC1 siRNA or expression of TSC2-HBD was also sufficient for stimulating Rac1 activity, suggesting that TCS2 interaction with TSC1 may be involved in the regulation of Rac1 activity. We conclude that TSC2 acts upstream of Rac1 in pathways regulating actin and focal adhesion remodeling.

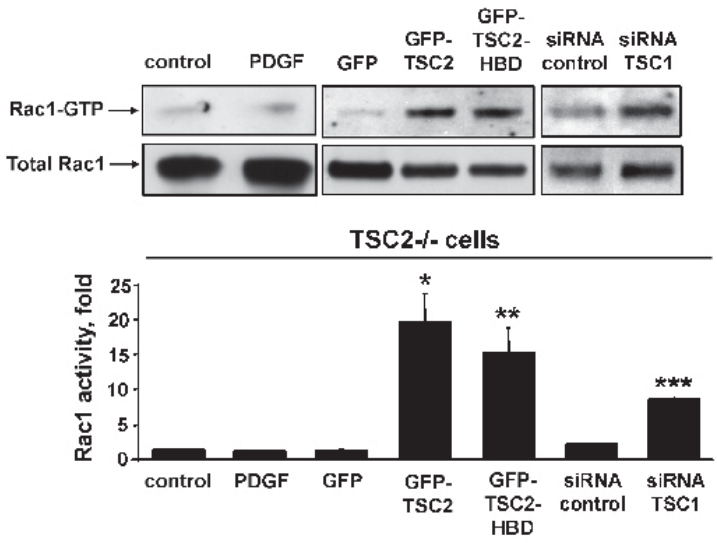


Figure 11. TSC2 is required for Rac1 activation. A: TSC2-/- cells were stimulated with 10 ng/ml of PDGF or diluent for 10 min or transfected with TSC2 constructs, siRNA TSC1 or control siRNA or GFP, followed by Rac1 activity assay[1].

The small GTPase RhoA is necessary for stress fiber and focal adhesion formation and TSC1 activates Rho. To determine whether TSC2-induced stress fiber disassembly was by impaired signaling downstream of Rho, we co-transfected TSC2 or TSC2-HBD with constitutively active Rho, V14Rho. In cells co-transfected with V14Rho stress fibers were maintained compared to cells expressing TSC2 alone. These data suggest that stress fiber

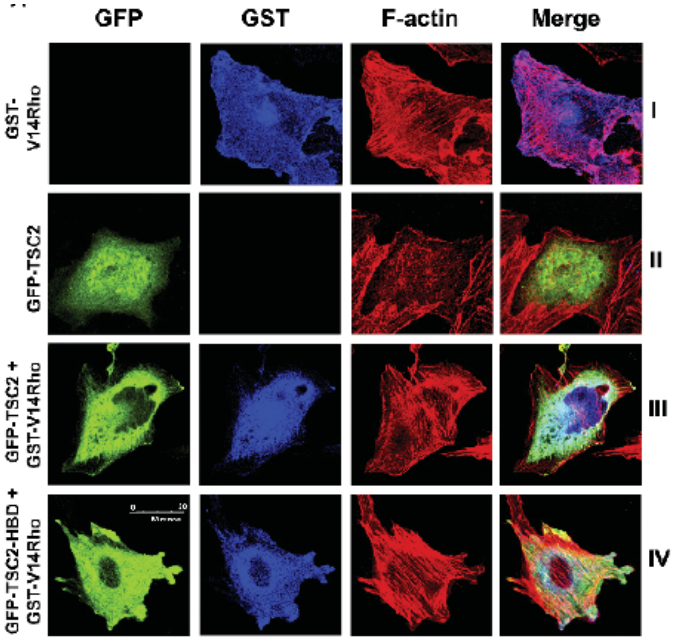


Figure 12. Constitutively active Rho rescues TSC2-induced stress fiber disassembly. TSC2-/- cells were transfected with pEBG-V12Rho; or GFP-TSC2; or GFP-TSC2 was co-transfected with pEBG-V12Rho plasmids; or pEGFP-TSC2-HBD was co-transfected with pEBG-V12Rho plasmid; then cells were stained with anti-GFP to detect GFP, GFP-TSC2, or GFP-TSC2-HBD (green), anti-GST to detect GST, or GST-V12Rho (blue), and rhodamine phalloidin to detect F-actin (red). Cells were analyzed using Leica TCS SP2 Confocal Microscope at 1000X magnification. Images are representative of three independent experiments. Scale bar is 20 microns.

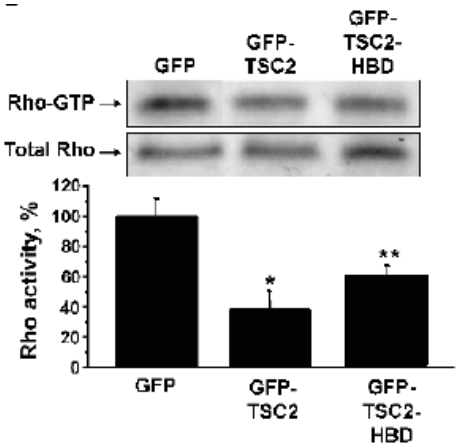


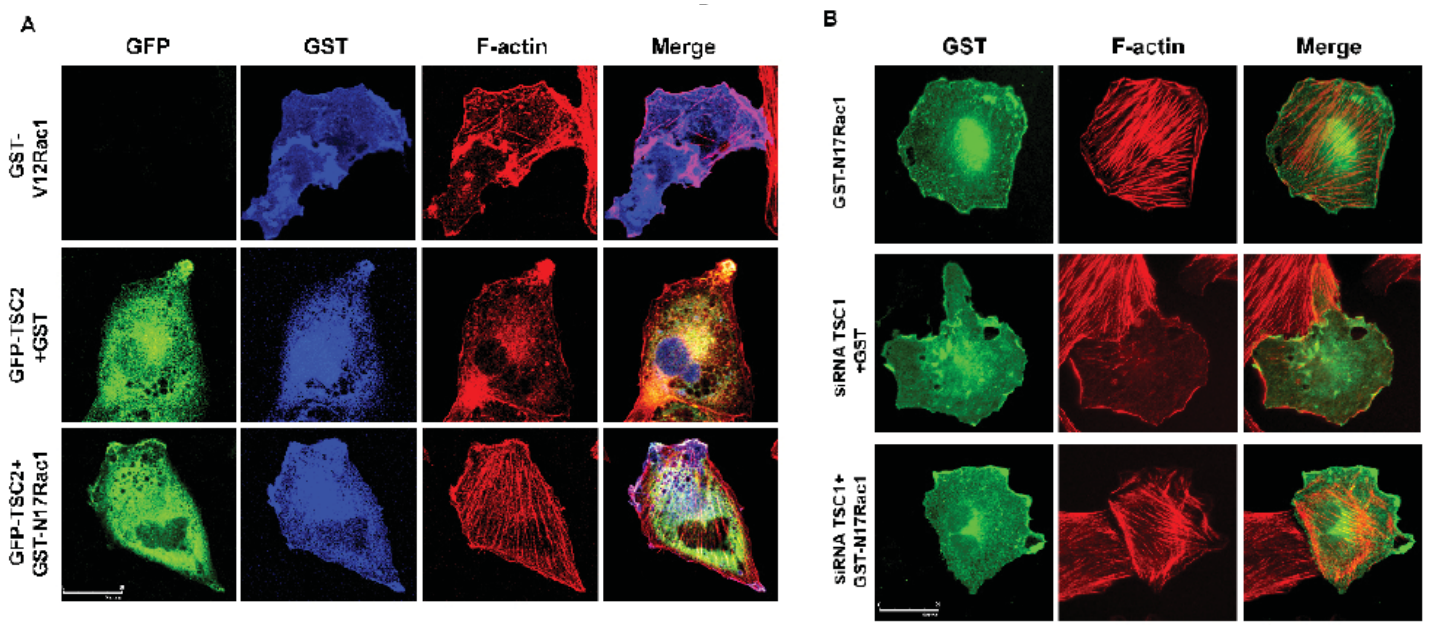
Figure 13. TSC2 and TSC2-HBD inhibit Rho activity in TSC2-/- cells. Cells were transfected with pEGFP-TSC2, pEGFP-TSC2-HBD, and control pEGFP plasmids, expressing GFP-tagged TSC2, GFP-TSC2-HBD, and control GFP, respectively, and then Rho activity assay was performed. Immunoblot analysis of Rho-GTP pull-down with Rhotekin-RBD agarose (top panel) and whole cell lysates (bottom panel) was performed with anti-Rho antibodies. Quantitative analysis of three independent experiments was performed using Gel-Pro Analyzer Software. *P < 0.001 for GFP-TSC2 vs. GFP; **P < 0.001 for GFP-TSC2-HBD vs. GFP by ANOVA (Bonferroni-Dunn test).

disassembly induced by TSC2 and TSC2-HBD is not due to failure in pathways downstream of Rho that regulate assembly and maintenance of stress fibers.

To investigate whether TSC2 may modulate Rho activity, we examined whether TSC2 expression affected endogenous Rho activity in TSC2-/- cells. TSC2 modestly but reproducibly attenuated Rho activity by 64 $\pm$ 13 % compared to GFP-transfected cells. TSC2-HBD alone was also sufficient for the attenuation of Rho activity by 40 $\pm$ 9 % compared to control GFP. Taken together, these results indicate that TSC2-induced actin rearrangements involve activation of Rac1 and inhibition of RhoA.

Rho and Rac1 both regulate stress fiber formation and focal adhesion remodeling in reciprocal manner, as such activation of Rac1 results in the inhibition of Rho, and vice versa. To clarify the hierarchy of Rho inhibition and Rac1 activation in TSC2-dependent stress fiber disassembly, we analyzed whether the activated form of Rac1, V12Rac1, could promote actin rearrangements in TSC2-/- cells; then we carried out co-transfection experiments of dominant negative GST-tagged Rac1 (N17Rac1) with TSC2. Expression of V12Rac1 promoted stress fiber disassembly in the cell center and formation of cortical actin at the cell periphery similar to the effect of TSC2.

In contrast, in cells co-expressing N17Rac1 and TSC2, stress fibers were maintained suggesting that TSC2-induced stress fiber disassembly requires Rac1 activation. Furthermore, when N17Rac1 was co-microinjected with siRNA TSC1, stress fibers also remained, indicating that TSC1-dependent stress fiber formation requires the negative regulation of Rac1 activity. Because stress fibers are maintained by active Rho, and inactive Rac1 co-expressed with TSC2 or siRNA TSC1 does not promote stress fiber disassembly, this data indicates that TSC2 requires activation of Rac1, followed by inhibition of Rho in regulating actin remodeling.



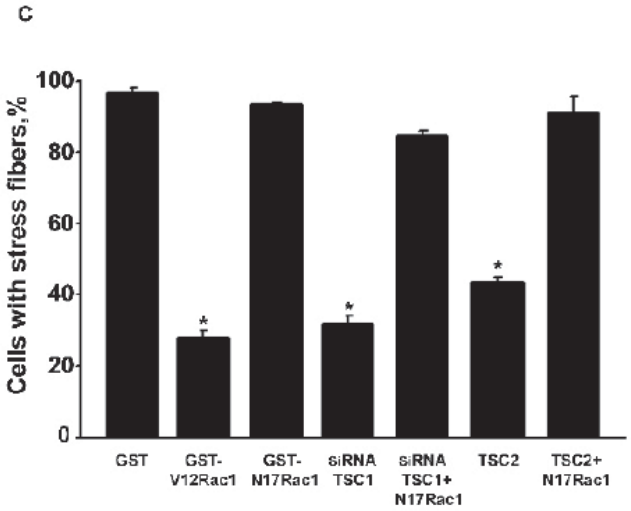


Figure 14. Dominant negative Rac1 abrogates TSC2- and siRNA TSC1-induced stress fiber disassembly. A: Serum-deprived cells were transfected with either pEBG-V12Rac1CA expressing activated Rac1, or the cells were co-transfected with pEGFP-TSC2 and pEBG-N17Rac1 plasmids, expressing GFP-TSC2 and GST-N17Rac1, respectively, were stained with anti-GFP to detect GFP-TSC2, (green), anti-GST to detect GST, GST-V12Rac1, GST-N17Rac1 (blue), and phalloidin rhodamine to detect F-actin (red), followed by analysis using a Leica TCS SP2 Confocal Microscope at 1000X magnification. Images are representative of three independent experiments. Scale bar is 20 microns. **B:** Cells, microinjected with GST-N17Rac1, co-microinjected with siRNA TSC1 and GST to identify microinjected cells, or co-microinjected with siRNA TSC1 and N17Rac1, were stained with anti-GST antibody to detect GST and GST-N1Rac1 (green), and phalloidin rhodamine to detect F-actin (red). Images are representative of three independent experiments. **C:** Quantitative analysis of F-actin staining. Data represent the percentage of cells with stress fibers per total number of cells transfected with plasmids expressing GST, GST-V12Rac1, GST-N17Rac1, GFP-TSC2, or co-expressed GFP-TSC2 and GST-N17Rac1, siRNA TSC1 and GST-N17Rac1, or siRNA TSC1 and control GST taken as 100%. Data represent the mean ± SE from 3 independent experiments. *P<0.001 for GST-V12Rac1 vs. GST, siRNA TSC1 + GST vs. siRNA TSC1 + GST-N17Rac1, GFP-TSC2 vs. GFP-TSC2 + GST-N17Rac1 by ANOVA (Bonferroni Dunn test).

Task 3. Determine whether phosphatidylinositol 3-kinase (PI3K) is an upstream activator of TSC1- and TSC2-dependent cell adhesion and motility

To test whether PI3K is involved in TSC1- and TSC2-dependent adhesion and motility, we examined whether PDGF, a well established PI3K activator will affect actin cytoskeleton and Rac1 activation. Surprisingly, PDGF, which promotes stress fiber disassembly and lamellapodia formation in most cell types, and promoted stress fiber disassembly in 3T3 cells, had little effect on actin rearrangements and lamellapodia formation in TSC2-/- cells (Fig. 15A). This was more surprising, because we previously demonstrated that PDGF stimulates migration of TSC2-/- ELT3 cells[3]. Stimulation of TSC2-/- cells with PDGF had little effect on the basal Rac1 activity (Fig. 11), while PDGF stimulated Rac activity in 3T3 cells (data not shown), which were used as a model cell line.

Since PDGF activates PI3K through activation of its specific PDGF receptor α (PDGFRα) and PDGFRβ, we next examined their expression in TSC2-/-. As seen in Figure 15B, immunoblot analysis revealed that PDGFRα and PDGFRβ are expressed in these cells, and TSC2 expression had little effect on both

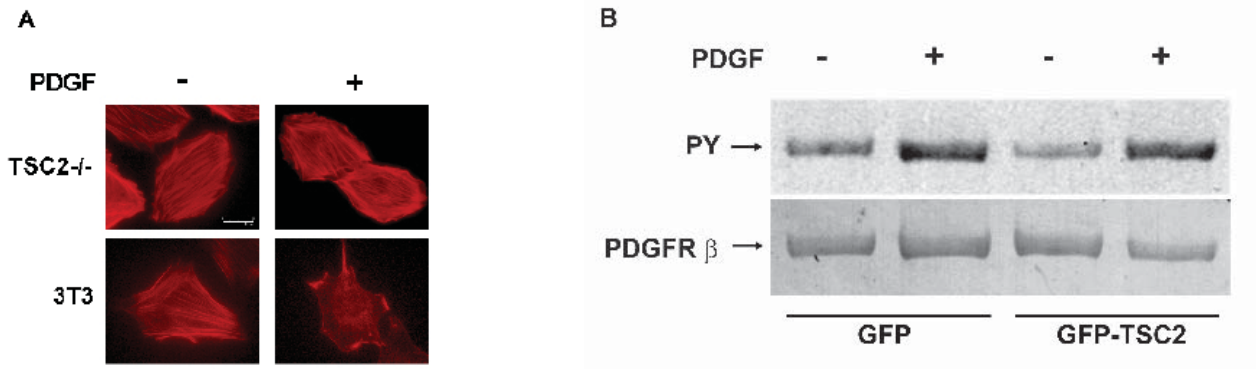


Figure 15. PDGF has little effect on stress fiber disassembly in TSC2-/- cells. A: Rhodamine phalloidin staining of F-actin of serum-deprived TSC2-/- and 3T3 cells, which were either stimulated with 10 ng/ml of PDGF (+) or diluent (-) for 10 minutes. **PDGF receptor activation in TSC2-/- cells.** Serum-deprived cells, transfected with either GFP or GFP-TSC2, were stimulated with PDGF, and equalized in protein content whole cell lysates were subjected to immunoprecipitation (IP) with either anti-PDGFRβ **(B)** or PDGFRα **(C)** antibodies; then immunoblot analysis was performed with either anti-PDGFRβ, PDGFRα, or anti-phosphotyrosine (PY) antibodies.

receptor levels. Stimulation of cells with PDGF-BB induced activation of PDGFRβ, but has little effect on PDGFRα. These data suggest that PDGF-induced signaling is not defective in rat TSC2-/- cells at the receptor levels.

Taken together our study demonstrates that TSC2 functions as a negative regulator of cell migration, and both TSC1 and TSC2 proteins regulate cell adhesion and motility. Thus, loss of TSC1 and/or TSC2 function which may contribute to pathological conditions associated with TSC.

References:

1 E.A. Goncharova, D.A. Goncharov, P.N. Lim et al., *Am. J. Respir. Cell Mol. Biol.* **34**, 473 (2006).

2 Elena Goncharova, Dmitry Goncharov, Daniel Noonan et al., *J. Cell Biol.* **167** (6), 1171 (2004).

3 C. Irani, E. Goncharova, D. Hunter et al., *Am. J. Physiol.* **282**, L854 (2002).

Key Research Accomplishments:

- TSC1/TSC2 tumor suppressor proteins regulate cell adhesion and motility;

- Loss of TSC2 function results in increased cell migration and invasiveness;

- Interaction between TSC1 and TSC2 is critical for TSC1-dependent activation of Rho GTPase;

- TSC1/TSC2 regulate activity of Rho GTPases Rac1 and RhoA;

- Our study identified novel function of tumor suppressor TSC2 in regulating activities of Rac1 and RhoA GTPases, focal adhesion formation and cell motility.

Reportable Outcomes:

Manuscripts and abstracts published and submitted for publication, which were supported by this award:

1. Goncharova E.A., Goncharov D.A., Noonan D., **V.P. Krymskaya**. TSC2 modulates actin cytoskeleton and cell adhesion through TSC1-binding domain and Rac1 GTPase. Journal of Cell Biology. 167: 1171-1182, 2004.

2. Goncharova, E.A., Goncharov, D.A., Lim, P.N., **V.P. Krymskaya.** Role of RhoA GTPase and tuberin in LAM-derived (LAMD) cell migration. Proceedings of the American Thoracic Society Vol. 2: A122, 2005.

3. **Krymskaya, V.P.** TSC/mTOR Control Actin Cytoskeleton: Dual Functionality Confirmed. LAM/TS International Research Conference, Cincinnati, OH. April, 2005.

4. Goncharova, E.A., Goncharov, D.A., Spaits, M., Noonan, D., Talovskaya, E., Eszterhas, A., and **V.P. Krymskaya.** Abnormal Growth of Smooth Muscle Cells in Lymphangioleiomyomatosis (LAM): Role for Tumor Suppressor TSC2. American Journal of Respiratory Cell and Molecular Biology 34(5): 561-72, May 2006.

5. Goncharova, E.A., Goncharov, D.A., Spaits, M., Lim, P.N., Noonan, D., and **V.P. Krymskaya**. Modulation of cell migration and invasiveness by tumor suppressor TSC2 in lymphangioleiomy omatosis. American Journal of Respiratory Cell and Molecular Biology 34(4): 473-80, Apr 2006.

6. “Regulation of actin cytoskeleton by TSC2,” Quest-2006 Meeting: Signaling New Treatments for Disease, p.9, 2006, Queenstown, New Zealand.

Additionally, the PI presented her studies supported by this award during platform presentations:

Apr, 2005 “TSC/mTOR Control Actin Cytoskeleton: Dual Functionality Confirmed,” LAM/TS International Research Conference, Cincinnati, OH.

Mar, 2006	"Signaling by Rho GTPases in TSC: TSC1/TSC2 as regulators of actin cytoskeleton and cell adhesion", PKD Foundation Meeting, Chicago, IL - Polycystic Kidney Disease and Tuberous Sclerosis Complex.
Mar, 2006	"LAM: From Cell Culture to Clinical Trials," Lecture for Pulmonary Fellows at the Pulmonary, Allergy & Critical Care Division at the University of Pennsylvania School of Medicine
Apr, 2006	"The Role of TSC1/TSC2 Tumor Suppressor Complex in LAM," Australian & New Zealand LAM Science Symposium, Blue Mountain, Australia.
May, 2006	"PI3K-TSC-mTOR Signaling Pathway in Cancer: Rationale and Promise," University of Auckland, New Zealand.
May, 2006	"TSC1/TSC2 m-TOR Signalling Pathway: Novel Regulator of Cell Growth and Proliferation,: University of Sydney, Pharmacy Faculty Seminar, Australia.
May, 2006	"Cell Signaling by Tumor Suppressor Complex TSC1/TSC2: From disease to cell function," CEET, University of Pennsylvania, Philadelphia, PA
May, 2006	"LAM Cells: Migration and Metastasis" Clinical and Fundamental Mechanisms in LAM: New Advances, American Thoracic Society International Conference, San Diego, CA
Jun, 2006	"Human LAM Cell Model: Experimental Paradigms and Limitations," Harvard Medical School, Boston, MA
Sep, 2006	"Regulation of actin cytoskeleton by TSC2," Quest 2006 Meeting, Signaling New Treatments for Disease, Queenstown, New Zealand

Conclusions:

Collectively, research supported by this grant have been highly productive and have provided new and exciting information about the molecular mechanism of TSC1 and TSC2 signaling and its potential role in TSC disease progression. We found that TSC2 functions as a negative regulator of cell migration, modulates actin dynamics and cell adhesion, and the TSC1-binding domain (TSC2-HBD) is essential for this function of TSC2. Expression of TSC2 or TSC2-HBD in TSC2-/- cells promoted Rac1 activation, inhibition of Rho, stress fiber disassembly, and focal adhesion remodeling. The down-regulation of TSC1 with TSC1 siRNA in TSC2-/- cells activated Rac1 and induced loss of stress fibers. Our data indicate that TSC1 inhibits Rac1 and TSC2 blocks this activity of TSC1. Since TSC1 and TSC2 regulate Rho and Rac1, whose activities are interconnected in a reciprocal fashion, loss of either TSC1 or TSC2 function may result in the deregulation of cell motility and adhesion, which are associated with the pathobiology of TSC.

Appendices

Appendix includes the following published manuscripts and abstracts, which were supported by this award:

1. Goncharova E.A., Goncharov D.A., Noonan D., **V.P. Krymskaya**. TSC2 modulates actin cytoskeleton and cell adhesion through TSC1-binding domain and Rac1 GTPase. Journal of Cell Biology. 167: 1171-1182, 2004.

2. Goncharova, E.A., Goncharov, D.A., Lim, P.N., **V.P. Krymskaya.** Role of RhoA GTPase and tuberin in LAM-derived (LAMD) cell migration. Proceedings of the American Thoracic Society Vol. 2: A122, 2005.

3. **Krymskaya, V.P.** TSC/mTOR Control Actin Cytoskeleton: Dual Functionality Confirmed. LAM/TS International Research Conference, Cincinnati, OH. April, 2005.

4. Goncharova, E.A., Goncharov, D.A., Spaits, M., Noonan, D., Talovskaya, E., Eszterhas, A., and **V.P. Krymskaya.** Abnormal Growth of Smooth Muscle Cells in Lymphangioleiomyomatosis (LAM): Role for Tumor Suppressor TSC2. American Journal of Respiratory Cell and Molecular Biology 34(5): 561-72, May 2006.

5. Goncharova, E.A., Goncharov, D.A., Spaits, M., Lim, P.N., Noonan, D., and **V.P. Krymskaya**. Modulation of cell migration and invasiveness by tumor suppressor TSC2 in lymphangioleiomyomatosis. American Journal of Respiratory Cell and Molecular Biology 34(4): 473-80, Apr 2006.

TSC2 modulates actin cytoskeleton and focal adhesion through TSC1-binding domain and the Rac1 GTPase

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Tuberous sclerosis complex (TSC) 1 and TSC2 are thought to be involved in protein translational regulation and cell growth, and loss of their function is a cause of TSC and lymphangioleiomyomatosis (LAM). However, TSC1 also activates Rho and regulates cell adhesion. We found that TSC2 modulates actin dynamics and cell adhesion and the TSC1-binding domain (TSC2-HBD) is essential for this function of TSC2. Expression of TSC2 or TSC2-HBD in TSC2−/− cells promoted Rac1 activation, inhibition of Rho, stress fiber disassembly, and focal adhesion remodeling. The down-regulation of TSC1 with TSC1 siRNA in TSC2−/− cells activated Rac1 and induced loss of stress fibers. Our data indicate that TSC1 inhibits Rac1 and TSC2 blocks this activity of TSC1. Because TSC1 and TSC2 regulate Rho and Rac1, whose activities are interconnected in a reciprocal fashion, loss of either TSC1 or TSC2 function may result in the deregulation of cell motility and adhesion, which are associated with the pathobiology of TSC and LAM.

Introduction

Tumor suppressors tuberous sclerosis complex (TSC) 1 and TSC2, also named as hamartin and tuberin, respectively, play a critical role in protein translational regulation and cell growth from *Drosophila melanogaster* to mammals (Krymskaya, 2003; Kwiatkowski, 2003; Manning and Cantley, 2003). TSC1 and TSC2 proteins form a cytosolic heterodimer and exert their function as negative regulators of the mammalian target of rapamycin (mTOR) signaling pathway (Nellist et al., 1999; Goncharova et al., 2002; Kwiatkowski et al., 2002). TSC2 encodes in its COOH terminus a GTPase-activating protein (GAP) for small GTPase Rheb (Ras homologue enriched in brain), whose activity antagonizes mTOR signaling (Gao et al., 2002; Garami et al., 2003; Inoki et al., 2003a; Li et al., 2004). Growth factors, insulin, nutrients, and the cellular energy levels regulate the activity of TSC2 (McManus and Alessi, 2002; Inoki et al., 2003b).

TSC1 (gene encoding protein TSC1, hamartin) and *TSC2* (gene encoding protein TSC2, tuberin) genes are susceptibility factors for TSC (Crino and Henske, 1999; Sparagana and Roach, 2000; Cheadle et al., 2000) and lymphangioleiomyomatosis (LAM; Sullivan, 1998; Carsillo et al., 2000; Johnson and Tattersfield, 2002). The pathobiology of TSC and LAM are generally thought to be linked to abnormal cell growth. However, the neurological manifestations of TSC have been defined as a "neuronal migration disorder" and occur due to aberrant neuronal motility during brain development (Crino and Henske, 1999; Vinters et al., 1999; Gutmann et al., 2000; Sparagana and Roach, 2000); and LAM is a potentially metastatic disease (Yu et al., 2001; Henske, 2003; Karbowniczek et al., 2003), suggesting a role for TSC1 and TSC2 in cell motility. Furthermore, TSC1-deficient murine embryonic fibroblasts have an impaired ability to form serum-induced stress fibers and focal adhesions (Kwiatkowski et al., 2002). Conversely, overexpression of TSC1 or TSC2 in human kidney epithelial cells results in increased E-cadherin expression, increased cell adhesion, and reduced chemotactic migration (Astrinidis et al., 2002; Li et al., 2003). Importantly, TSC1 binds to the ezrin-radixin-moesin (ERM) family of actin-binding proteins (Lamb et al., 2000). In cultured cortical neurons, TSC1 physically anchors intermediate filaments to the actin cytoskeleton by binding to both neurofilament light chains and the ERM proteins (Haddad et al., 2002). Together, these data suggest the potential involvement of TSC2 and TSC1 in cell motility. However, the precise mechanism and

The online version of this article includes supplemental material.

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Abbreviations used in this paper: ERM, ezrin-radixin-moesin; GAP, GTPase-activating protein; LAM, lymphangioleiomyomatosis; LAMD, human LAM-derived; mTOR, mammalian target of rapamycin; PDGFR, PDGF receptor; S6K, p70 S6 kinase; SE, standard error; TSC, tuberous sclerosis complex; TSC2-HBD, TSC1-binding domain of TSC2.

The Journal of Cell Biology, Vol. 167, No. 6, December 20, 2004 1171–1182
http://www.jcb.org/cgi/doi/10.1083/jcb.200405130

Supplemental Material can be found at:
http://www.jcb.org/cgi/content/full/jcb.200405130/DC1

the relevance of these findings to aberrant neuronal motility in TSC and LAM metastasis remains an enigma.

The Rho family of small GTPases, RhoA, Rac, and Cdc42, are key regulators of actin cytoskeletal remodeling, cell adhesion, and migration. RhoA promotes the formation of stress fibers that are linked to focal adhesions; Rac induces the formation of membrane ruffles and lamellipodia; and Cdc42 induces filopodia formation (Etienne-Manneville and Hall, 2002; Burridge and Wennerberg, 2004). Reciprocal activation of RhoA, Rac, and Cdc42 is critical for the regulation of cell adhesion and motility (Horwitz and Parsons, 1999; Etienne-Manneville and Hall, 2002), and dysregulation of this balance promotes cell transformation and metastasis (Sahai and Marshall, 2002). Recent studies suggest that TSC1 regulates Rho activity through the Rho-activating domain within its NH_2 terminus by an unknown mechanism (Lamb et al., 2000). Interestingly, the Rho-activating domain of TSC1 (amino acids 145–510) overlaps with the domain that binds TSC2: the amino acids 302–430 of TSC1 (Hodges et al., 2001) associate with amino acids 1–418 of TSC2 and are required for TSC1–TSC2 complex formation, which potentially stabilizes each protein (Nellist et al., 1999; Henske, 2003; Krymskaya and Shipley, 2003). These data suggest that the interaction of TSC1 with TSC2 may be important for TSC1-dependent Rho activation and cell adhesion. However, how TSC2 and TSC1 complex formation is involved in regulating actin remodeling and adhesion has not been identified.

Here, we show that TSC2 regulates the actin cytoskeleton and focal adhesion, and the TSC1-binding domain of TSC2 (TSC2-HBD), which corresponds to amino acids 1–460 in the NH_2 terminus of TSC2, is both necessary and sufficient for this function. Importantly, down-regulation of TSC1 with siRNA in TSC2−/− cells also induces disassembly of stress fibers and focal adhesion remodeling, indicating that TSC1 is required for TSC2-dependent actin remodeling. Furthermore, we show that the role of TSC2 in modulating actin dynamics is distinct from its function as a negative regulator of the rapamycin-sensitive mTOR/p70 S6 kinase (S6K) signaling pathway.

Results

TSC2 is necessary for dynamic membrane protrusions during wound closure

To explore whether or not TSC2 regulates cell motility, we performed live imaging of the wound closure of TSC2-deficient

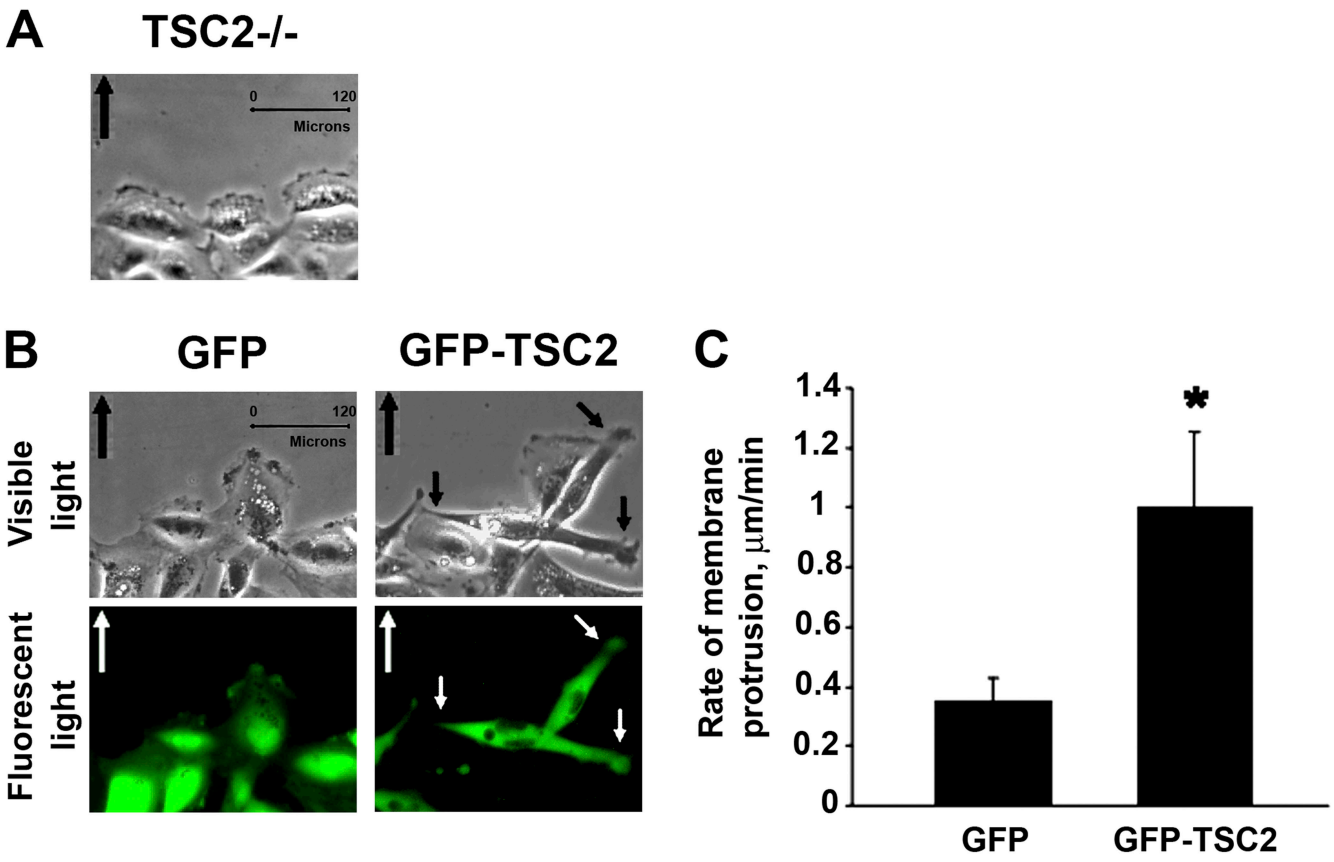


Figure 1. **Re-expression of TSC2 changes TSC2−/− cell morphology and dynamics during wound closure.** (A) Phase-contrast micrograph of time-lapse analysis of TSC2−/− cell motility during wound closure at 4 h after wound scraping; images are representative of three independent experiments. (B) Phase-contrast and fluorescence micrographs demonstrate the representative phenotypes of GFP- or GFP-TSC2–infected cells. TSC2−/− cells, infected with GFP-TSC2 or control GFP replication-deficient adenovirus constructs, were first serum-deprived, and then subjected to live image analysis of wound closure in the presence of 2% FBS. Short arrows indicate differences in membrane protrusion; long arrows indicate direction of cell movement. Bars, 120 μm. Images were taken using a Leitz Inverted Microscope in both the phase-contrast and green fluorescence channels. Images are representative from three independent experiments. (C) Statistical analysis of the rate of membrane protrusion in TSC2−/− cells infected either with GFP or GFP-TSC2. *, $P < 0.0001$ for GFP-TSC2-infected cells versus GFP-infected cells by ANOVA (Bonferroni-Dunn test).

smooth muscle ELT3 cells, which were either untreated or transduced with a replication-deficient adenovirus expressing GFP-tagged wild-type TSC2 or control GFP. As shown in Fig. 1 A and Video 1 (available at http://www.jcb.org/cgi/content/full/jcb.200405130/DC1), TSC2−/− cells retained their motile properties (Irani et al., 2002); however, their movement was characterized by the formation of short lamellipodia at the leading edge of migrating cells. Expression of GFP-tagged TSC2 markedly changed the pattern of cell dynamics; during wound closure, moving cells formed dynamic membrane protrusions, and the rate of membrane extension for GFP-TSC2–infected cells was 1.00 ± 0.25 μm min^{-1} compared with 0.35 ± 0.08 μm min^{-1} for GFP-infected cells (Fig. 1, B and C; and Videos 2 and 3, available at http://www.jcb.org/cgi/content/full/jcb.200405130/DC1). Expression of control GFP in TSC2−/− cells had little effect on the pattern of cell movement (Fig. 1 B and Videos 4 and 5, available at http://www.jcb.org/cgi/content/full/jcb.200405130/DC1). These data demonstrate that reexpression of TSC2 markedly changes TSC2−/− cell morphology during cell motility and suggest that TSC2 might be important for the formation of membrane protrusions during directional movement.

TSC2 and TSC2-HBD regulate stress fiber disassembly

Because cell motility is regulated by actin remodeling, we examined actin rearrangements in TSC2−/− cells. F-actin staining revealed abundant stress fiber formation (Fig. 2 A, top). Surprisingly, PDGF, which promotes stress fiber disassembly and lamellipodia formation in most cell types (Zigmond, 1996) and promoted stress fiber disassembly in 3T3 cells (Fig. 2 A, bottom), had little effect on actin rearrangements and lamelli-

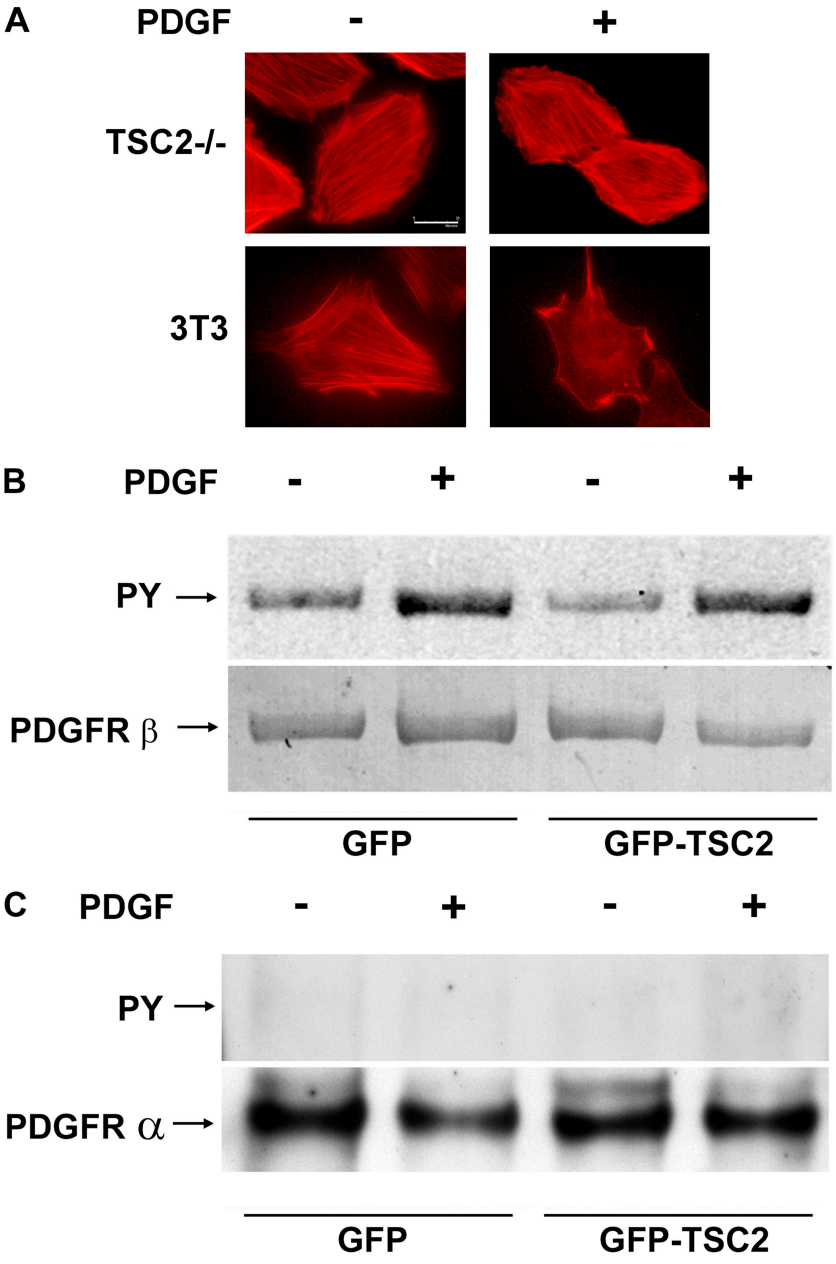


Figure 2. **PDGF has little effect on stress fiber disassembly in TSC2−/− cells.** (A) Rhodamine phalloidin staining of F-actin of serum-deprived TSC2−/− and 3T3 cells, which were either stimulated with 10 ng/ml of PDGF (+) or diluent (−) for 10 min. PDGFR activation in TSC2−/− cells: serum-deprived cells, transfected with either GFP or GFP-TSC2, were stimulated with PDGF. Equalized in protein content whole cell lysates were subjected to immunoprecipitation with either anti-PDGFRβ (B) or PDGFRα (C) antibodies; and then immunoblot analysis was performed with either anti-PDGFRβ, PDGFRα, or anti-phosphotyrosine (PY) antibodies.

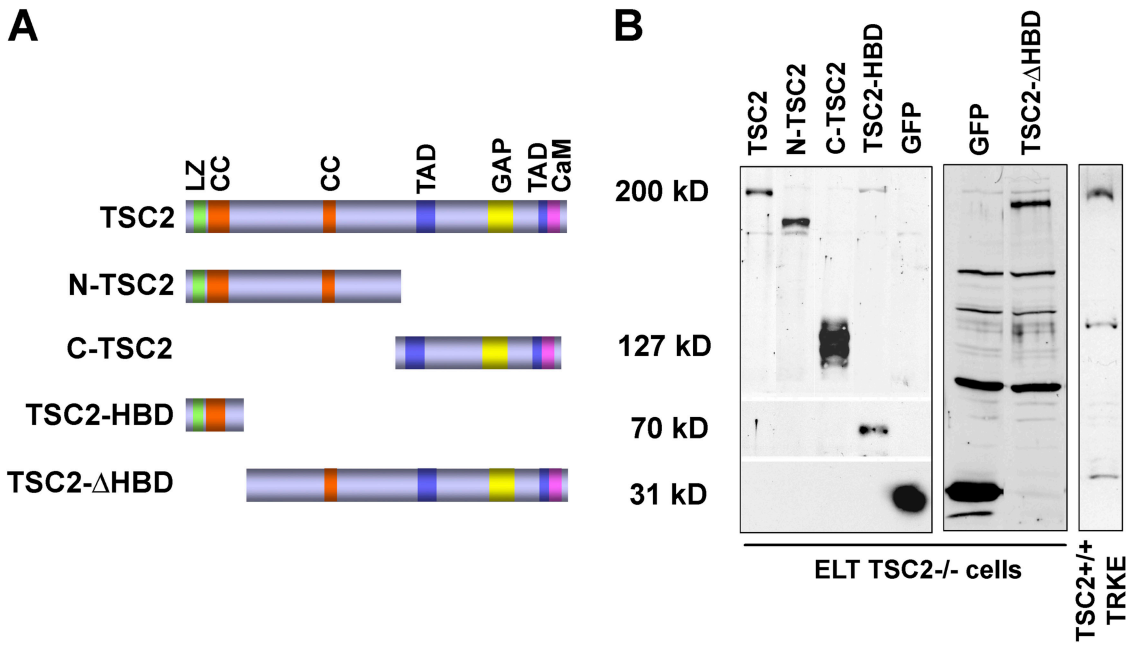


Figure 3. **Schematic representation and expression of GFP-tagged TSC2 constructs in the TSC2−/− cells.** (A) TSC2 includes leucine zipper (LZ), two coiled-coiled (CC), two transcription-activating domains (TAD), GAP homology (GAP), and calmodulin (CaM)-binding domain. (B) To identify expression of TSC2 mutants, cells were transfected with pEGFP vectors expressing GFP-tagged TSC2, N-TSC2, C-TSC2, TSC2-HBD, TSC2-ΔHBD, or control GFP. After 24 h of transient transfection, cells were lysed, and whole cell lysates were subjected to 8% SDS-PAGE followed by immunoblot analysis with anti-GFP antiserum. TSC2-positive rat TRKE cells, used as a positive control, were lysed and subjected to 8% SDS-PAGE followed by immunoblot analysis with anti-TSC2 antibody.

podia formation in TSC2−/− cells (Fig. 2 A, top). This finding was more surprising because we previously demonstrated that PDGF stimulates migration of TSC2−/− ELT3 cells (smooth muscle cells derived from Eker rat uterine leiomyomas; Irani et al., 2002). Because Zhang et al. (2003) demonstrated that PDGF receptor (PDGFR) α and PDGFRβ levels were reduced in TSC2−/−TP53−/− murine embryonic fibroblasts, we examined their expression in TSC2−/− rat ELT3 cells. Immunoblot analysis revealed that PDGFRα and PDGFRβ are expressed in these cells, and TSC2 expression had little effect on both receptor levels (Fig. 2, B and C). Stimulation of cells with PDGF-BB induced activation of PDGFRβ, but has little effect on PDGFRα (Fig. 2, B and C, respectively). These data suggest that PDGF-induced signaling is not defective in rat TSC2−/− cells at the receptor levels.

To examine whether TSC2 is required for actin remodeling, and to identify which domain of TSC2 is important for stress fiber disassembly, we tested a panel of GFP-tagged deletion constructs of TSC2 (Fig. 3). The reexpression of full-length TSC2 in TSC2−/− cells markedly promoted stress fiber disassembly and the formation of cortical actin compared with cells transfected with control GFP (Fig. 4 A). Interestingly, the expression of TSC2-ΔHBD in TSC2−/− cells containing the Rheb GAP domain (Fig. 4 A) or the expression of C-TSC2 (not depicted) had little effect on stress fiber disassembly. In contrast, expression of N-TSC2 (not depicted) or TSC2-HBD was sufficient to induce stress fiber disassembly (Fig. 4 A).

Stress fiber assembly is regulated by activation of Rho GTPase, which could be activated by TSC1 through its Rho-activating domain (Lamb et al., 2000). Because TSC2 binds TSC1 (Nellist et al., 1999, 2001; Hodges et al., 2001) by the domain that overlaps the Rho-activating domain of TSC1 and potentially inhibits TSC1-dependent Rho activation, we investigated whether or not stress fiber disassembly involves TSC1. As seen in Fig. 4 A, microinjection of siRNA in TSC2−/− cells directed against TSC1 promoted stress fiber disassembly similar to the effects of the expression of TSC2 or TSC2-HBD. Because TSC2-ΔHBD had no effect on stress fiber disassembly, but TSC2-HBD and siRNA TSC1 were sufficient to produce this effect in TSC2−/− cells, these data suggest that TSC2-dependent actin remodeling involves TSC1.

Quantitative analysis of F-actin staining revealed that 94.6 ± 1.7% of GFP-transfected cells showed stress fibers; in contrast, TSC2, TSC2-HBD, or siRNA TSC1 promoted marked stress fiber disassembly by 42.3 ± 1.9%, 52.7 ± 1.5%, and 31.7 ± 2.6%, respectively (Fig. 4 B). Because TSC2 is a susceptibility factor for LAM disease, we tested whether TSC2 or TSC2-HBD will affect actin remodeling in primary cultures of cells derived from the LAM tumors (Goncharova et al., 2002). As seen in Fig. 4 C, TSC2 and TSC2-HBD expression also promoted stress fiber disassembly in human LAM-derived (LAMD) cells, suggesting that TSC2 might be important for abnormal metastatic cell growth associated with TSC2 deficiency in LAM (Henske, 2003).

TSC2 or TSC2-HBD promotes focal adhesion remodeling

Because actin rearrangements are accompanied by focal adhesion remodeling, we examined whether or not TSC2-induced stress fiber disassembly correlates with changes in focal adhe-

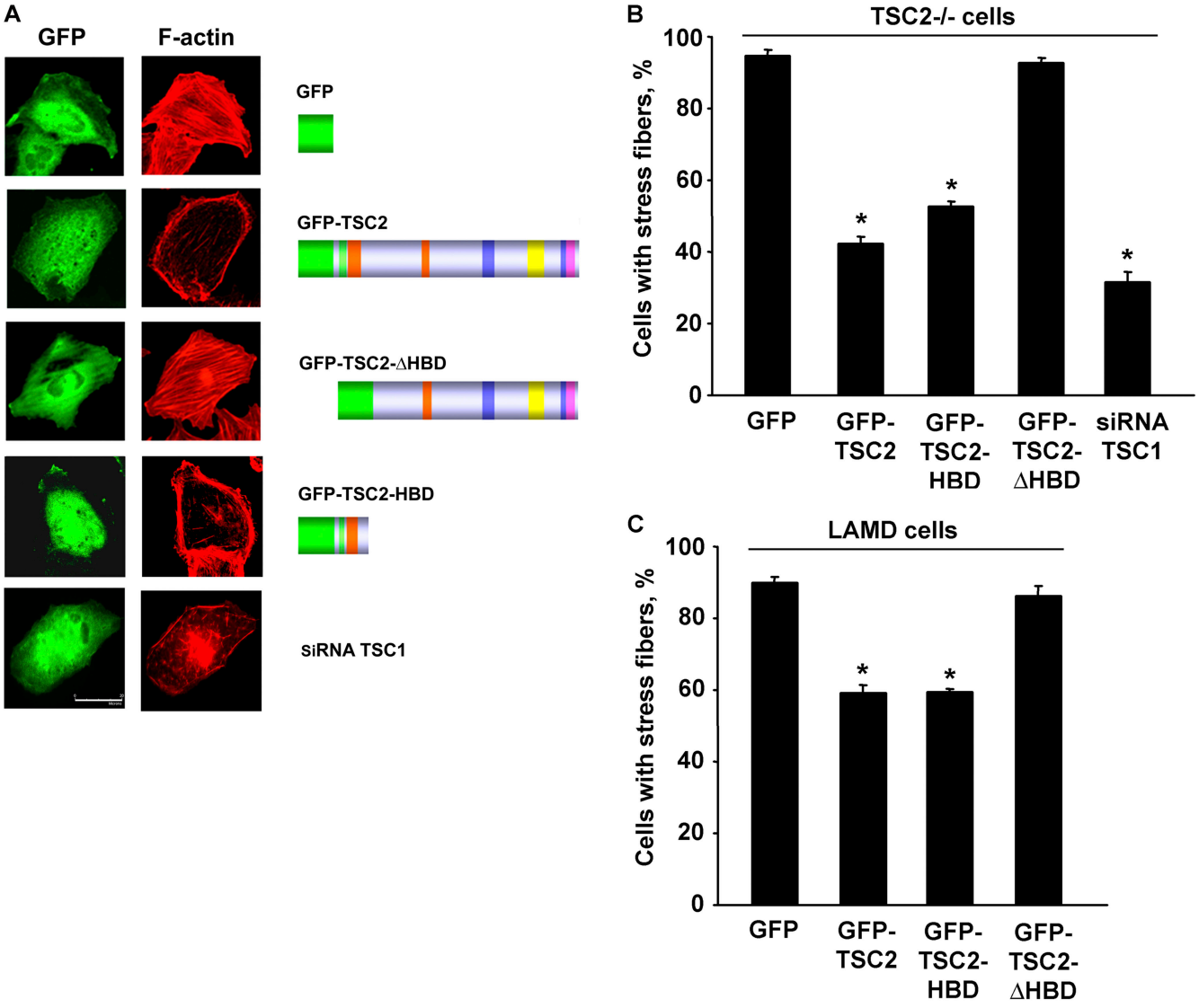


Figure 4. **TSC2, TSC2-HBD, and siRNA TSC1 promote stress fiber disassembly.** (A, left) F-actin staining (red) of TSC2−/− cells transfected with GFP-TSC2 or the indicated GFP-tagged TSC2 constructs identified by immunostaining with anti-GFP antibody (green) or microinjected with siRNA TSC1 and GST to identify injected cells (green). (right) Schematic representation of TSC2 constructs. Bar, 20 μm. (B) Quantitative analysis of F-actin staining. Data represent the percentage of cells with stress fibers per total number of cells transfected with pEGFP, pEGFP-TSC2, pEGFP-TSC2-HBD, or pEGFP-TSC2-ΔHBD taken as 100%. A total of 205 of GFP-TSC2–transfected, 360 of GFP-TSC2-HBD–transfected, 302 of GFP-TSC2-ΔHBD–transfected, 331 of GFP-transfected, and 644 siRNA TSC1-microinjected cells were analyzed from three independent experiments. Data represent the mean ± SE. *, $P < 0.0001$ for GFP-TSC2–, GFP-TSC2-HBD–transfected cells, or siRNA TSC1 + GST-microinjected cells versus GFP-transfected cells by ANOVA (Bonferroni-Dunn test). (C) Statistical analysis of F-actin staining of LAMD cells transfected with GFP, GFP-TSC2, GFP-TSC2-HBD, and GFP-TSC2-ΔHBD. Data represent the mean ± SE from two independent experiments. *, $P < 0.001$ for GFP-TSC2– and GFP-TSC2-HBD–transfected cells versus GFP-transfected cells by ANOVA (Bonferroni-Dunn test).

sion formation. Immunohistochemical analysis with anti-vinculin antibody showed that in TSC2−/− cells focal adhesions were localized throughout the cell as well as at the cell periphery (Fig. 5 A, top; and Video 6, available at http://www.jcb.org/cgi/content/full/jcb.200405130/DC1). Expression of TSC2 promoted marked changes in the shape and size of the focal adhesions: most focal adhesions in the center of the cells were disassembled, and the quantity of focal adhesions per cell was attenuated by 30.3 ± 5.9% compared with control. At the same time, the size of focal adhesions at the cell periphery was also markedly decreased (Fig. 5 A, middle; and Video 7, available at http://www.jcb.org/cgi/content/full/jcb.200405130/DC1). Importantly, TSC2-HBD, which is involved in TSC1–TSC2 complex formation, also promoted focal adhesion disassembly in the center of the cells (Fig. 5 A, bottom; and Video 8, available at http://www.jcb.org/cgi/content/full/jcb.200405130/DC1). TSC1 regulates cell adhesion, and its inactivation results in the loss of focal adhesion (Lamb et al., 2000). To determine if focal adhesion remodeling in TSC2−/− cells requires TSC1, we microinjected siRNA TSC1 and found that down-regulation of TSC1 promoted focal adhesion disassembly in TSC2−/− cells (Fig. 5 B). These results demonstrate that expression of TSC2-HBD and the down-regulation of TSC1 with siRNA TSC1 are sufficient for focal adhesion disassembly, which suggest that TSC2 involves TSC1 in regulating focal adhesion remodeling.

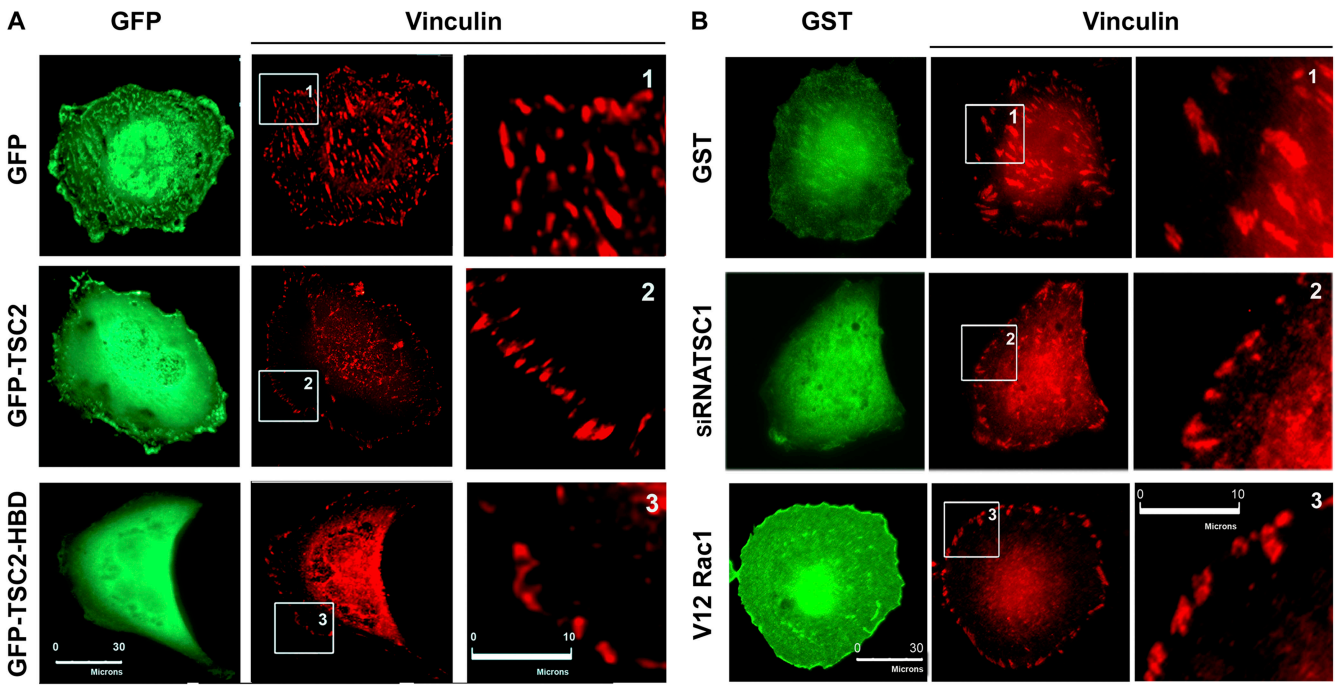
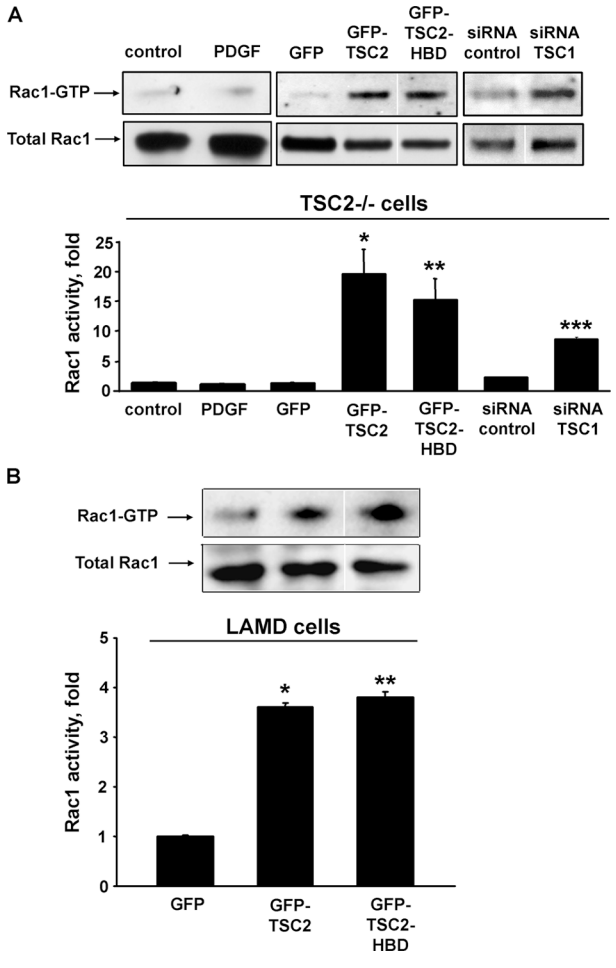


Figure 5. **TSC2, TSC2-HBD, and siRNA TSC1 modulate focal adhesion formation.** (A) Cells were transfected with control pEGFP (top), pEGFP-TSC2 (middle), or pEGFP-TSC2-HBD (bottom) plasmid, and then immunostained with anti-GFP (green) and anti-vinculin (red) antibodies to detect focal adhesions. Images are representative of three separate experiments. (B) Cells were transfected with control GST, GST-tagged V12Rac1, or siRNA TSC1 comicroinjected with GST to identify microinjected cells, followed by immunostaining with anti-GST (green) and anti-vinculin (red) antibodies. Representative images from 138 microinjected cells. Changes in the shape and size of the focal adhesion are represented in enlarged insets (1–3). Bars: (A and B) 30 μm; (insets) 10 μm.



Because the cortical actin staining observed in TSC2-transfected cells was reminiscent of the effects of Rac1 (Etienne-Manneville and Hall, 2002) and because small GTPase Rac1 induces the formation of membrane ruffles and lamellipodia, which ultimately results in focal adhesion remodeling, we examined the effect of the constitutively active form of Rac1 (V12Rac1) on focal adhesion formation in TSC2−/− cells. Vinculin immunostaining showed that V12Rac1 expression induced marked changes in focal adhesion formation similar to effects of TSC2 (Fig. 5 B), suggesting a potential link between TSC2 and Rac1 signaling.

Figure 6. **TSC2, TSC2-HBD, and siRNA TSC1 activate Rac1.** (A) TSC2−/− cells were stimulated with 10 ng/ml of PDGF or diluent for 10 min or transfected with plasmids expressing GFP-tagged TSC2 or TSC2-HBD, or GFP as a control, or microinjected with siRNA TSC1 or control siRNA, and then subjected to the Rac1 activity assay. (top) Immunoblot analysis with anti-Rac1 antibody to detect Rac1 in pull-down assay with PAK-1 PBD agarose (top images) and in whole cell lysates (bottom images). Images are representative of three separate experiments. (bottom) Quantitative analysis of Rac1 activity assays using Gel-Pro Analyzer Software. Rac1 activity in cells transfected with control pEGFP plasmid was taken as a onefold. *, $P < 0.001$ for GFP-TSC2 versus GFP; **, $P < 0.001$ for GFP-TSC2-HBD versus GFP; ***, $P < 0.001$ for siRNA TSC1 versus control siRNA. Data represent the mean ± SE from three independent experiments. (B) LAMD cells were transfected with GFP-TSC2, GFP-TSC2-HBD, or GFP as a control, and then Rac1 activity assay was performed. (top) Immunoblot analysis with anti-Rac1 antibody to detect Rac1 in pull-down assay with PAK-1 PBD agarose (top images) and in whole cell lysates (bottom images). Images are representative of two separate experiments. (bottom) Quantitative analysis of Rac1 activity. Data represent the mean ± SE from two independent experiments. *, $P < 0.001$ for GFP-TSC2 versus GFP; **, $P < 0.001$ for GFP-TSC2-HBD versus GFP by ANOVA (Bonferroni-Dunn test). White lines indicate that intervening lanes have been spliced out.

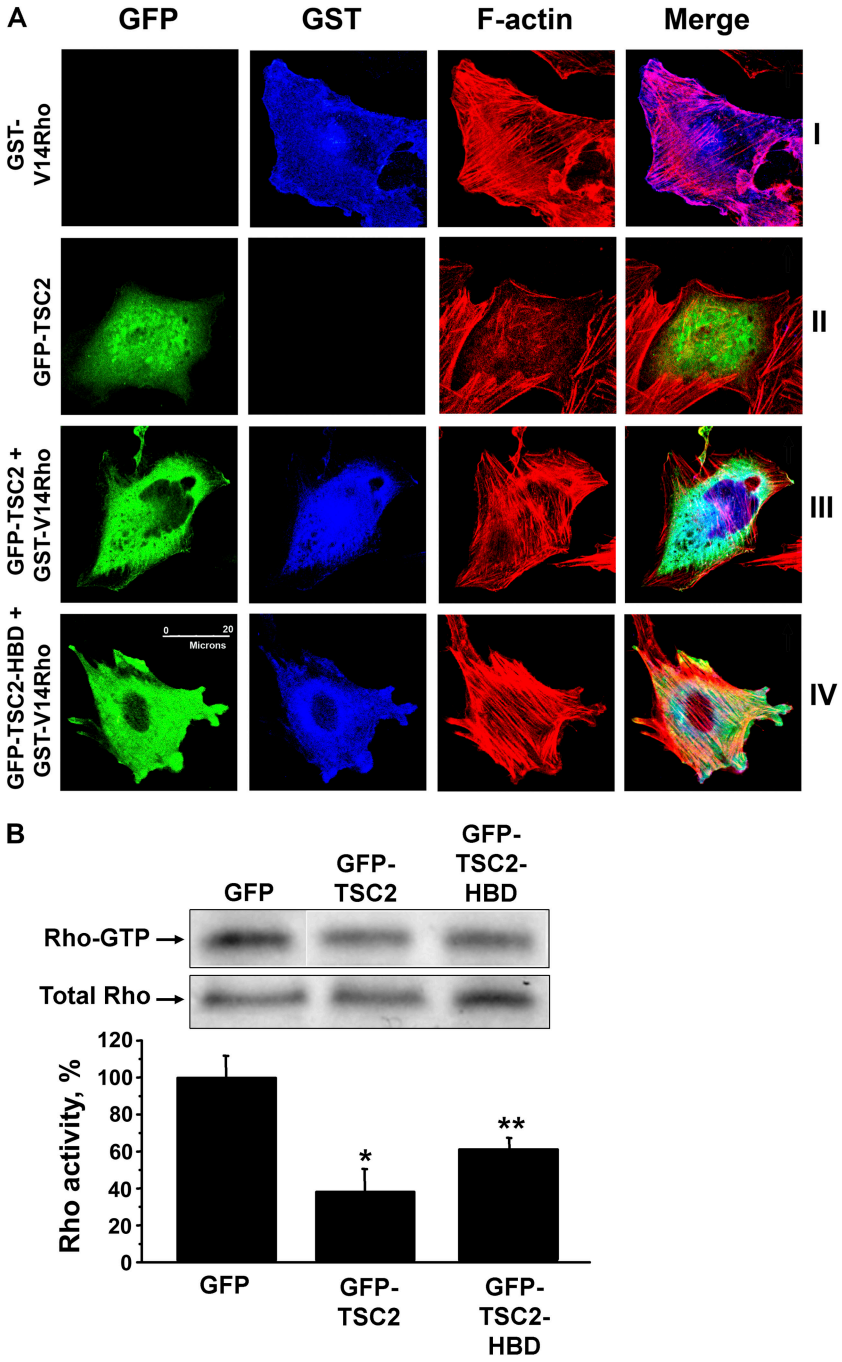


Figure 7. **TSC2 and TSC2-HBD inhibit Rho activity in TSC2−/− cells.** (A) TSC2−/− cells were transfected with pEBG-V12Rho or GFP-TSC2; GFP-TSC2 was cotransfected with pEBG-V12Rho plasmids; or pEGFP-TSC2-HBD was cotransfected with pEBG-V12Rho plasmid; and then cells were stained with anti-GFP to detect GFP, GFP-TSC2, or GFP-TSC2-HBD (green), anti-GST to detect GST, or GST-V12Rho (blue) and rhodamine phalloidin to detect F-actin (red). Images are representative of three independent experiments. Bar, 20 μm. (B) Cells were transfected with pEGFP-TSC2, pEGFP-TSC2-HBD, and control pEGFP plasmids expressing GFP-tagged TSC2, GFP-TSC2-HBD, and control GFP, respectively, and then Rho activity assay was performed. Immunoblot analysis of Rho-GTP pull-down with Rhotekin-RBD agarose (top) and whole cell lysates (bottom) was performed with anti-Rho antibodies. White lines indicate that intervening lanes have been spliced out. Quantitative analysis of three independent experiments was performed using Gel-Pro Analyzer Software. *, $P < 0.001$ for GFP-TSC2 versus GFP; **, $P < 0.001$ for GFP-TSC2-HBD versus GFP by ANOVA (Bonferroni-Dunn test).

Activation of Rac1 by TSC2

To clarify the function of Rac1 in TSC2-induced stress fiber disassembly and focal adhesion remodeling, we next examined Rac1 activation in TSC2−/− cells. Stimulation of TSC2−/− cells with PDGF had little effect on the basal Rac1 activity (Fig. 6 A), whereas PDGF-stimulated Rac1 activity in 3T3 cells (not depicted), which were used as a model cell line. By expressing TSC2 in TSC2−/− cells, we found that TSC2 alone was sufficient to markedly increase Rac1 activity compared with cells transfected with control GFP (Fig. 6 A). Similarly, Rac1 activity was increased in LAMD cells transfected with TSC2, indicating that TSC2 may elicit activation of Rac1 (Fig. 6 B). Importantly, down-regulation of TSC1 with TSC1 siRNA or expression of TSC2-HBD was also sufficient for stimulating Rac1 activity (Fig. 6 A), suggesting that TCS2 interaction with TSC1 may be involved in the regulation of Rac1 activity. We conclude that TSC2 acts upstream of Rac1 in pathways regulating actin and focal adhesion remodeling.

TSC2 and TSC2-HBD inhibit Rho activity

The small GTPase RhoA is necessary for stress fiber and focal adhesion formation (Etienne-Manneville and Hall, 2002) and TSC1 activates Rho (Lamb et al., 2000). To determine whether or not TSC2-induced stress fiber disassembly was by impaired signaling downstream of Rho, we cotransfected TSC2 or TSC2-HBD with constitutively active Rho, V14Rho. As seen

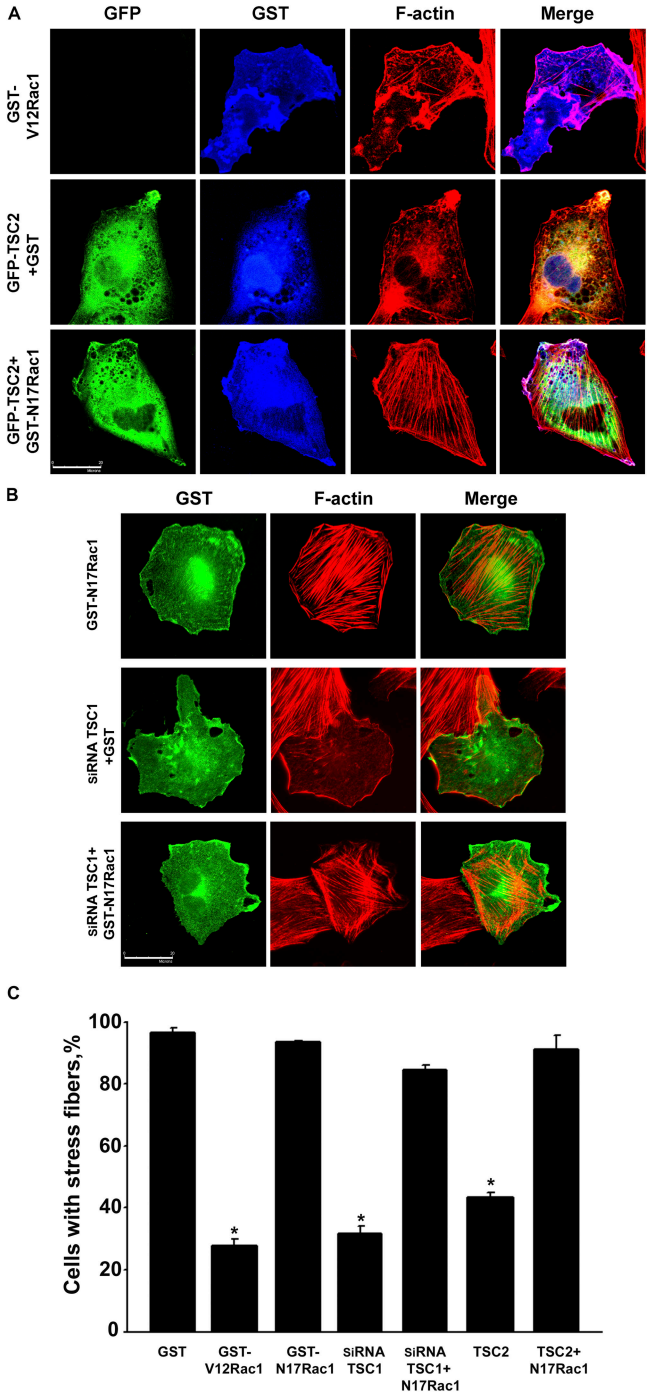


Figure 8. **Dominant-negative Rac1 abrogates TSC2- and siRNA TSC1-induced stress fiber disassembly.** (A) Serum-deprived cells were transfected with pEBG-V12Rac1 expressing activated Rac1 or the cells were cotransfected with pEGFP-TSC2 and pEBG-N17Rac1 plasmids, expressing GFP-TSC2 and GST-N17Rac1, respectively, and were stained with anti-GFP to detect GFP-TSC2 (green), anti-GST to detect GST, GST-V12Rac1, GST-N17Rac1 (blue), and phalloidin rhodamine to detect F-actin (red). Images are representative of three independent experiments. Bar, 20 μm. (B) Cells, microinjected with GST-N17Rac1, comicroinjected with siRNA TSC1 and GST to identify microinjected cells, or comicroinjected with siRNA TSC1 and N17Rac1, were stained with anti-GST antibody to detect GST and GST-N1Rac1 (green) and phalloidin rhodamine to detect F-actin (red). Images are representative of three independent experiments. (C) Quantitative analysis of F-actin staining. Data represent the percentage of cells with stress fibers per total number of cells transfected with plasmids expressing GST, GST-V12Rac1, GST-N17Rac1, GFP-TSC2, or coexpressed GFP-TSC2 and GST-N17Rac1, siRNA TSC1 and GST-N17Rac1, or siRNA TSC1 and control GST taken as 100%. Data represent the mean ± SE from three independent experiments. *, $P < 0.001$ for GST-V12Rac1 versus GST, siRNA TSC1 + GST versus siRNA TSC1 + GST-N17Rac1, and GFP-TSC2 versus GFP-TSC2 + GST-N17Rac1 by ANOVA (Bonferroni-Dunn test).

in Fig. 7 A (panels III and IV, respectively), in cells cotransfected with V14Rho, stress fibers were maintained compared with cells expressing TSC2 alone (Fig. 7 A, panel II). These data suggest that stress fiber disassembly induced by TSC2 and TSC2-HBD is not due to failure in pathways downstream of Rho that regulate assembly and maintenance of stress fibers.

To investigate whether or not TSC2 may modulate Rho activity, we examined if TSC2 expression affected endogenous Rho activity in TSC2−/− cells. As seen in Fig. 7 B, TSC2 modestly but reproducibly attenuated Rho activity by 64 ± 13% compared with GFP-transfected cells. TSC2-HBD alone was also sufficient for the attenuation of Rho activity by 40 ± 9% compared with control GFP (Fig. 7 B). Together, these results indicate that TSC2-induced actin rearrangements involve activation of Rac1 and inhibition of Rho.

Rac1 acts upstream of Rho in TSC2-dependent actin remodeling

Rho and Rac1 both regulate stress fiber formation and focal adhesion remodeling in a reciprocal manner, as such activation of Rac1 results in the inhibition of Rho and vice versa (Horwitz and Parsons, 1999). To clarify the hierarchy of Rho inhibition and Rac1 activation in TSC2-dependent stress fiber disassembly, we analyzed whether or not the activated form of Rac1, V12Rac1, could promote actin rearrangements in TSC2−/− cells; and then we performed cotransfection experiments of dominant-negative GST-tagged Rac1 (N17Rac1) with TSC2. Expression of V12Rac1 promoted stress fiber disassembly in the cell center and formation of cortical actin at the cell periphery similar to the effect of TSC2 (Fig. 8 A, top and middle, respectively). In contrast, in cells coexpressing N17Rac1 and TSC2, stress fibers were maintained (Fig. 8 A, bottom), suggesting that TSC2-induced stress fiber disassembly requires Rac1 activation. Furthermore, when N17Rac1 was comicroinjected with siRNA TSC1, stress fibers also remained (Fig. 8 B, bottom), indicating that TSC1-dependent stress fiber formation requires the negative regulation of Rac1 activity. Quantitative analysis of these experiments is presented in Fig. 8 C. Because stress fibers are maintained by active Rho, and inactive Rac1 coexpressed with TSC2 or siRNA TSC1 does not promote stress fiber disassembly, this data indicates that TSC2 requires activation of Rac1, followed by inhibition of Rho, in regulating actin remodeling.

TSC2-HBD is not required for the negative regulation of ribosomal protein S6 activation

To address the relationship between the role that TSC2 plays in regulating actin dynamics and its function as a negative regulator of protein translation through mTOR, we examined the effect of

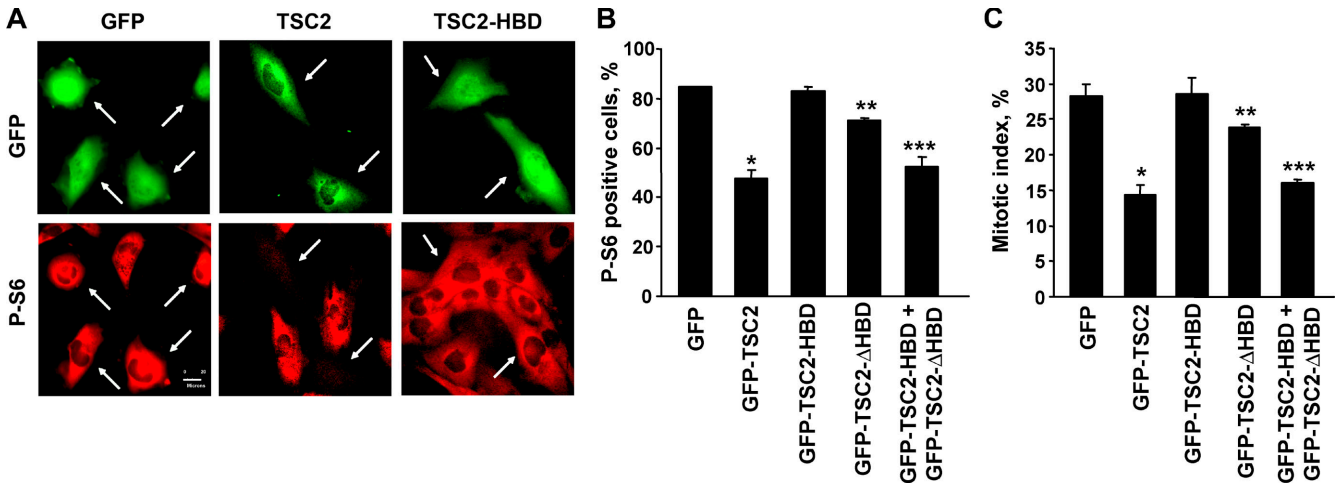


Figure 9. **Expression of TSC2, but not TSC2-HBD, inhibits ribosomal protein S6 phosphorylation and DNA synthesis in TSC2−/− cells.** TSC2−/− cells were transfected with plasmids expressing GFP-tagged TSC2, TSC2-ΔHBD, or TSC2-HBD, or comicroinjected with pEGFP-TSC2-HBD and pEGFP-TSC2-ΔHBD plasmids, serum-deprived for 24 h, and immunostained with anti–phospho-S6 (red) antibody and anti-GFP antiserum (green); or BrdU incorporation analysis was performed. (A) Representative images of three separate experiments were taken. Arrows indicate transfected cells. (B) Quantitative analysis of P-S6 immunostaining. Data represent the percentage of P-S6–positive transfected cells per total number of transfected cells. *, $P < 0.001$ for GFP-TSC2 versus GFP; **, $P < 0.001$ for GFP-TSC2-ΔHBD versus GFP; ***, $P < 0.001$ for GFP-TSC2-HBD + GFP-TSC2-ΔHBD versus GFP. (C) BrdU incorporation analysis of transfected cells. Mitotic index represents the percentage of BrdU-positive transfected cells compared with the total number of transfected cells. Data are mean ± SE of three separate experiments. *, $P < 0.001$ for GFP-TSC2 versus GFP; **, $P < 0.001$ for GFP-TSC2-ΔHBD versus GFP; ***, $P < 0.001$ for GFP-TSC2-HBD + GFP-TSC2-ΔHBD versus GFP.

rapamycin, a specific mTOR inhibitor, on the actin cytoskeleton in the TSC2−/− cells at a dose that completely abrogates S6K activity. We found that rapamycin had little effect on actin dynamics (unpublished data), which serves as evidence that the regulation of cell dynamics is a novel function of TSC2, which is independent from its function in protein translational regulation.

Because TSC2 negatively regulates the activity of ribosomal protein S6 by inhibition of its phosphorylation (Goncharova et al., 2002), we investigated whether TSC2-HBD or TSC2-ΔHBD affects ribosomal protein S6 hyperphosphorylation in TSC2−/− cells. Consistent with previously published results (Goncharova et al., 2002), full-length TSC2 inhibited S6 phosphorylation by 56.2 ± 3.9% compared with GFP-transfected cells (Fig. 9, A and B). TSC2-ΔHBD also significantly, however to a lesser extent than full-length TSC2, inhibited S6 phosphorylation by 22.7 ± 2.8%. Importantly, comicroinjection of TSC2-HBD and TSC2-ΔHBD inhibited phospho-S6 by 47.4 ± 3.3%, which was comparable to the inhibitory effect of full-length TSC2 (Fig. 9 B). In contrast, expression of TSC2-HBD had little effect on S6 phosphorylation, suggesting that this domain of TSC2 is not required for the regulation of ribosomal protein S6 activation (Fig. 9, A and B).

In parallel, we examined the effect of TSC2 mutants on DNA synthesis. As we previously demonstrated (Goncharova et al., 2002), TSC2, but not TSC2-HBD, significantly inhibited TSC2−/− cell proliferation by 49.4 ± 4.7%. Importantly, TSC2-ΔHBD alone was sufficient for inhibition of DNA synthesis, however, to a much lesser extent than full-length TSC2. By coexpressing TSC2-HBD and TSC2-ΔHBD we found that DNA synthesis was inhibited by 43.2 ± 1.4%, which was comparable to inhibition by full-length TSC2 (Fig. 9 C). Collectively, these data demonstrate that TSC2-HBD is not required for S6 phosphorylation and regulation of DNA synthesis.

Discussion

TSC1 has been identified as an activator of Rho and a regulator of cell adhesion (Lamb et al., 2000). The Rho-activating domain of TSC1 overlaps with the region that binds TSC2 (Nellist et al., 1999, 2001; Hodges et al., 2001), indicating that TSC2 may modulate TSC1-dependent activation of Rho. Our work identifies a novel function of TSC2 as a modulator of the actin cytoskeleton and focal adhesion remodeling. In our model, TSC1 inhibits Rac1, and TSC2 blocks this activity of TSC1 that is a prerequisite to the activation of Rac1 and the subsequent inhibition of Rho; this, in turn, promotes stress fiber disassembly and focal adhesion remodeling (Fig. 10). Loss of function of either TSC1 or TSC2 due to inactivating mutations potentially promotes deregulation of the TSC1–TSC2 complex formation followed by deregulation of Rac1 and Rho activities, which, consequently, induces abnormal cell motility associated with the pathobiology of LAM and TSC.

Actin dynamics is a major cellular process regulating cell morphogenesis, movement, and behavior. In the normal cellular context, directional cell movement requires the formation of membrane protrusions and new focal adhesions, and stabilization of existing adhesions, which are regulated by Rho GTPases. Activity of these GTPases is modulated by a plethora of signaling molecules among which TSC2 appears as an important physiological modulator. Activation of Rac1 by reexpression of TSC2 in TSC2−/− cells resulted in the reorganization of the actin cytoskeleton and focal adhesion remodeling. Through attenuation of Rho activity, TSC2 facilitates stress fiber disassembly and focal adhesion remodeling, thus consequently promoting dynamic membrane protrusions. Our work indicates that TSC2 functions as a modulator of Rac1 and Rho activation and actin remodeling, which involves TSC1. TSC1, a

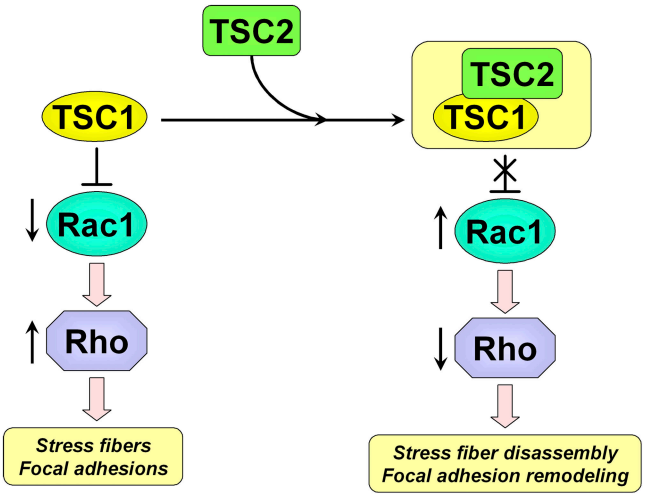


Figure 10. **Schematic representation of the TSC2-dependent modulation of actin cytoskeleton and focal adhesion.** TSC1 inhibits Rac1, and TSC2 blocks this activity of TSC1. TSC2 through its specific TSC1-binding domain forms a complex with TSC1, which is a prerequisite for Rac1 activation and Rho inhibition. This, in turn, promotes stress fiber disassembly and focal adhesion remodeling. Dysregulation of TSC2 function due to inactivating mutations promotes deregulation of the TSC1–TSC2 complex formation, followed by deregulating Rac1 and Rho activation, which, in turn, results in abnormal cell motility and adhesion associated with LAM and TSC pathobiology.

binding partner of TSC2 (Nellist et al., 1999; Henske, 2003; Krymskaya and Shipley, 2003), regulates the activity of Rho and associates with the cortical proteins ERM, which serve as molecular bridges between the plasma membrane and the cortical actin (Lamb et al., 2000). The fact that TSC2 binds TSC1 through TSC2-HBD (van Slegtenhorst et al., 1998; Benvenuto et al., 2000; Hodges et al., 2001), which overlaps with the Rho-activating domain of TSC1 (Lamb et al., 2000), suggests the critical importance of the TSC2–TSC1 interaction for TSC1-dependent Rho activation and cell adhesion. Our data show that Rho can be inhibited by the reexpression of TSC2, which contradicts a previous paper by Astrinidis et al. (2002) in which the stable expression of TSC2 in the same TSC2−/− cells results in Rho activation. The possible reason for the discrepancy between our data and the previous paper lies within the potential differences in the mode of cell motility that differ in their requirement for Rho activation (Martin, 2003; Sahai and Marshall, 2003) due to differences in experimental approaches. Using transient TSC2 expression by mammalian expression vector or adenoviral infection in TSC2−/− cells, we show that TSC2 promotes "classical" lamellipodial or mesenchymal motility driven by activated Rac1 and the down-regulation of Rho activation. In contrast to this classical type of motility, some tumor cell lines require Rho activation to promote a rounded bleb-associated mode of motility (Sahai and Marshall, 2003). Astrinidis et al. (2002) use ELT3 and MDCK cell clones with stable overexpression of TSC2. We can't rule out that cell lines stably overexpressing TSC2 had changed their phenotype with concomitant alterations in the mode of motility that requires Rho activation as it is demonstrated by Astrinidis et al. (2002). Our data also show that Rac1 acts upstream of Rho in TSC1–TSC2–dependent actin remodeling. How TSC1–TSC2 activates Rac1 and inhibits Rho remains to be elucidated. One possibility is that TSC1–TSC2 may modulate the activity of kinases regulating Rac1/RhoGDI association (DerMardirossian et al., 2004). Additionally, differential cellular localization of TSC2 and its mutants (unpublished data) suggests that TSC2 functions in regulating Rac1 activity or in the complex formation with TSC1 may depend on the subcellular localization of TSC2, TSC1, and Rac1. Another avenue to explore in elucidating the relationship between TSC1–TSC2 and Rac1 is to determine the modulation of growth factor–stimulated Rac1 activity by TSC1–TSC2.

TSC2 regulates cell growth and proliferation through the mTOR/S6K signaling pathway, which is sensitive to rapamycin, thus inhibiting mTOR. In TSC2−/− cells, we determined that actin remodeling was insensitive to rapamycin, suggesting that the rapamycin-sensitive mTOR pathway does not contribute to biochemical events that occur as a part of the regulatory mechanisms of cell dynamics. Further support for this observation was provided by the phosphorylation level of ribosomal protein S6, which was unaffected by expression of TSC2-HBD, a domain of TSC2, which was necessary and sufficient to regulate Rac1/Rho activities and actin remodeling. Importantly, TSC2-ΔHBD, which contains the GAP homology domain, is not involved in TSC2-dependent actin rearrangement but is sufficient for the modulation of mTOR/S6K activity, which suggests that different domains of TSC2 are involved in the regulation of cell dynamics and protein translation/cell growth through activation of two independent signaling pathways. Because TSC2 is an upstream regulator of mTOR, and in yeast TOR controls the actin cytoskeleton (Schmelzle and Hall, 2000), further studies are needed to address the question of whether or not the rapamycin-insensitive component of mTOR is involved in TSC2-dependent actin dynamics.

Our current findings provide evidence that TSC2 plays an important role in regulating cell dynamics. Notably, the function of TSC2-HBD, which is essential for the TSC2-dependent regulation of the actin cytoskeleton and focal adhesion, is not required for the regulation of S6 phosphorylation and cell growth, indicating that TSC2 may influence both cell dynamics and protein translation. It is important to note that activation of Rac1 is required for neurite outgrowth (Kozma et al., 1997; Yamaguchi et al., 2001). In contrast, Rho activation promotes neurite retraction and an inhibition of neurite outgrowth (Katoh et al., 1998; Yamaguchi et al., 2001). Because the loss of TSC1 or TSC2 functions promotes aberrant neuronal motility during brain development (Crino and Henske, 1999; Vinters et al., 1999; Gutmann et al., 2000; Sparagana and Roach, 2000), TSC1 and TSC2 loss of functions may deregulate Rac and Rho activities, which may then contribute to TSC pathology. Because TSC and LAM disease severity is predominantly associated with the loss of TSC2 function, the perturbed balance between TSC2 and TSC1 interaction could be a key event in the pathobiology of TSC and LAM.

Materials and methods

Cell culture

LAMD and TSC2−/− ELT3 cells were derived from the Eker rat uterine leiomyoma (Howe et al., 1995) and maintained as previously described (Goncharova et al., 2002). TSC2−/− ELT3 cells were a gift from C.L.

Walker (National Institute of Environmental Health Sciences Center for Research on Environmental Disease, Smithville, TX). 3T3 fibroblasts were purchased from American Type Culture Collection (CCL-92) and maintained in DME supplemented with 10% FBS. All experiments were performed on serum-deprived cells before experiments for 24 h.

Plasmid and adenovirus construction

The pEGFP-TSC2, encoding GFP-tagged TSC2; pEGFP-N-TSC2, encoding 1–1113 amino acids of TSC2; and pEGFP-C-TSC2, encoding 1114–1784 amino acids of TSC2 mammalian expression constructs were created as previously described (Finlay et al., 2004). The pEGFP-TSC2-HBD or pEGFP-TSC2-ΔHBD plasmids, expressing 1–460 amino acids or 461–1784 amino acids of TSC2, respectively, were produced by digesting the pEGFP-TSC2 plasmid, expressing wild-type human TSC2, with SalI and FspI or FspI and XbaI endonucleases, respectively. These fragments were ligated with pEGFP-C3 vector digested with SmaI and SalI or SmaI and XbaI, respectively. Successful insertion of TSC2-HBD or TSC2-ΔHBD into pEGFP plasmid was confirmed by analytical digest and sequence analysis with pEGFP-C Sequencing Primer (BD Biosciences). Recombinant adenovirus expressing GFP and TSC2 cDNA constructs were created using the AdEasy vector system as described previously (Finlay et al., 2004). The expression of GFP-tagged TSC2 mammalian expression constructs and adenoviruses was confirmed by transient transfection or adenovirus infection of TSC2−/− cells with these vectors and immunoblot analysis of whole cell lysates with anti-GFP antibody (Fig. 3).

Transient transfection and replication-deficient adenovirus infection

Transient transfection was performed using the Effectene transfection reagent (QIAGEN) according to the manufacturer's protocol. Infection with replication-deficient adenovirus was performed as described previously (Lanuti et al., 1999). Transfection and virus infection were used in parallel and yielded similar results regarding the regulation of cell cytoskeleton, motility, and the modulation of Rac1 and Rho GTPases activities. pEBG-V14Rho, pEBG-N17Rac1, and pEBG-V12Rac1 expression vectors were a gift from M.M. Chou (University of Pennsylvania, Philadelphia, PA).

Wound assay and live imaging of wound closure

Cells, plated on chamberslides, were wounded by scraping a 10-μl pipette tip through the cell monolayer, and then gently washed with PBS, incubated with fresh media supplemented with 2% FBS for 2 h, followed by supravital analysis. Supravital analysis was performed in the micro-incubator (model CSMI; Harvard Apparatus) with constant 37°C temperature on an inverted microscope (model TE300; Nikon) equipped with a digital video camera (model Evolution QEi; Media Cybernetics) under 100 magnification for 8.3 h. Images were taken every 10 min in both the phase-contrast and fluorescence channels and were analyzed using Image-Pro Plus 5.0.0.39 software (Media Cybernetics).

Immunocytochemistry

Cells were washed three times with PBS, fixed with 3.7% PFA for 15 min, treated with 0.1% Triton X-100 for 30 min at RT, and blocked with 0.5% TSA Fluorescein System blocking reagent (NEN Life Science Products) in TBS. After incubation with rhodamine phalloidin (Molecular Probes) or primary and then secondary antibodies conjugated with either Alexa Fluor488, Alexa Fluor594, or Alexa Fluor633 (see online supplemental materials) cells were mounted in Vectashield mounting medium (Vector Laboratories). Immunostaining was analyzed using the scanning laser confocal microscopic system (model TCS SP2; Leica), a microscope (model Eclipse TE2000-E; Nikon) equipped with a digital video camera (model Evolution QEi; Media Cybernetics), or a microscope (model Eclipse E400; Nikon) equipped with a digital camera (model Coolpix 995; Nikon) under 1,000 magnification. Three-dimensional analysis was performed using Z-series images taken with z interval 0.1 μm, which were then three-dimensionally deconvoluted using AutoDebur + AutoVisualize Software 9.3 (AutoQuant Imaging, Inc.).

Immunoblot analysis

Serum-deprived TSC2−/− cells were stimulated with 10 ng/ml of PDGF-BB (Calbiochem) for 10 min followed by immunoblot analysis with anti-PDGFRβ (Santa Cruz Biotechnology, Inc.), anti-PDGFRα, and antiphosphotyrosine antibodies (Upstate Cell Signaling Solutions) as previously described (Goncharova et al., 2002).

Rac and Rho activity assays

Cells were transfected with pEGFP-TSC2, pEGFP-TSC2-HBD, pEGFP-TSC2-ΔHBD, control pEGFP plasmid, siRNA TSC1, or siGLO RISC-Free siRNA (Dharmacon Research, Inc.) as a control and growth arrested, and then Rho or Rac activities were measured using Rac or Rho Activation Assay Kits (Upstate Cell Signaling Solutions) according to the manufacturer's protocol. Rho and Rac1 were detected using anti-Rho (A, B, or C) or anti-Rac1 antibodies, respectively (Upstate Cell Signaling Solutions). siRNA TSC1 sequences were a gift from R. Lamb (Institute for Cancer Research, London, UK); anti-TSC1 antibody was a gift from M. Nellist (Erasmus University, Rotterdam, Netherlands).

Microinjection

Microinjection was performed using Eppendorf Microinjection System as described previously (Goncharova et al., 2002). 18 h after injection, cells were subjected to immunocytochemical or BrdU incorporation assays.

BrdUrd incorporation

Cells, transfected with plasmids expressed GFP-conjugated TSC2, TSC2-HBD, TSC2-ΔHBD, or GFP as a control, or coinjected with plasmids expressed TSC2-HBD and TSC2-ΔHBD, were maintained for 24 h in serum-free medium, and BrdU incorporation was assessed (Goncharova et al., 2002). The mitotic index was defined as the percentage of BrdU-positive transfected/injected cells per field/total number of transfected/injected cells per field.

Statistical analysis

Statistical analysis of F-actin staining or immunostaining was performed by using microscope (model Eclipse E400; Nikon) images taken at 200 magnification followed by quantitative analysis using Gel-Pro Analyzer Software. Data points from individual assays represent the mean values ± standard error (SE). Statistically significant differences among groups were assessed with the analysis of variance (ANOVA; Bonferroni-Dunn test), with values of $P < 0.05$ sufficient to reject the null hypothesis for all analyses. All experiments were designed with matched control conditions within each experiment to enable statistical comparison as paired samples.

Online supplemental material

Down-regulation of TSC1 level by siRNA TSC1 and primary and secondary antibodies used for immunocytochemical analysis are listed in supplemental materials. The corresponding movie files of live cells are organized as follows: Video 1 for Fig. 1 A and Videos 2–5 for Fig. 1 B. Movie files demonstrating three-dimensional projection of cells immunostained with anti-vinculin antibody are organized as follows: Videos 6–8 for Fig. 5. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200405130/DC1.

We thank Dr. Margaret M. Chou for helpful discussions, critical reading of the manuscript, and gift of pEBG-V14Rho, pEBG-N17Rac1, and pEBG-V12Rac1 expression vectors; Dr. Michael F. Olson for helpful discussions and critical reading of the manuscript; Dr. Cheryl L. Walker for the gift of TSC2−/− ELT3 cells; Dr. Richard Lamb for the gift of siRNA TSC1 sequences; Dr. Mark Nellist for the gift of anti-TSC1 antibody; Andrew Eszterhas for help with cell image software and equipment settings; and Dr. Reynold A. Panettieri for support and encouragement.

This work was supported by National Institutes of Health grants HL071106 (to V.P. Krymskaya) and HL67321 (to D. Noonan), Department of Defense Idea Development Award TS030012 (to V.P. Krymskaya), the Lymphangioleiomyomatosis Foundation (to V.P. Krymskaya and D. Noonan), and the University of Pennsylvania Research Fund (to V.P. Krymskaya).

Submitted: 21 May 2004
Accepted: 10 November 2004

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[A42] [Poster: G67] Role of RhoA GTPase and Tuberin in LAM-Derived (LAMD) Cell Migration

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Lymphangioleiomyomatosis (LAM) is a genetic disorder characterized by abnormal proliferation, migration, and differentiation of smooth-muscle-like cells within the lung. LAM progression correlates with somatic mutations and dysfunction of the tumor suppressor genes *TSC1* and *TSC2,* hamartin and tuberin, respectively. While the etiology of LAM remain unknown, clinical and genetic evidence support a metastatic model for LAM. We found that LAMD cells have high activity of RhoA GTPase, increased stress fiber formation, and increased basal (unstimulated) motility in the absence of any stimuli. Importantly, expression of tuberin abrogated the basal migration of LAMD cells. Further, the N-terminal, but not C-terminal region of tuberin was sufficient to inhibit cell migration. Expression of tuberin also inhibited Rho activity, promoted stress fiber disassembly and focal adhesion remodeling in LAMD cells. Inhibition of Rho and ROCK activities with Tat-C3 toxin, or specific ROCK inhibitors Y-27632 and HA-1077, respectively, decreased basal LAMD cell migration. Collectively our data demonstrate that LAMD cells have marked basal migratory activity and activated RhoA, which are abrogated by expression of tuberin. These data suggest that tuberin-dependent dysregulation of RhoA activity in LAMD cells may contribute to abnormal cell migration in LAM.

Date: Sunday, May 22, 2005
Session Info: [**] Thematic Poster Session (Abstract Page: A122) Session: 8:15 am-4:15 pm, INTERSTITIAL AND DIFFUSE LUNG DISEASE I
Presentation Time: 08:15 AM
Room: Area G (Hall C, Ground Level), San Diego Convention Center

TSC/mTOR Control Actin Cytoskeleton: Dual Functionality Confirmed

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TSC1 and TSC2 are generally thought to be involved in protein translational regulation and cell growth, and loss of their function is a cause of lymphangioleiomyomatosis (LAM) and tuberous sclerosis complex (TSC). Development of these diseases is generally linked to abnormal cell growth. However, LAM is a potentially metastatic disease and the neurological manifestations of TSC have been defined as a neurological migratory disorder, suggesting a role for TSC1 and TSC2 in cell motility. TOR, a downstream effector of TSC1/TSC2 has well established role in regulation of actin cytoskeleton in yeast; in mammalians, however this function of mTOR remained an enigma. Recently, two studies show that mTOR, as a part of rapamycin-insensitive mTOR complex 2 (mTORC2) regulate actin cytoskeleton [1,2]. Regulation of actin polymerization by growth factors and amino acids suggested that mTOR-dependent actin remodeling may be regulated by tumor suppressor proteins TSC1/TSC2. Our studies demonstrated that in TSC2-/- cells actin remodeling was insensitive to rapamycin suggesting that the rapamycin-sensitive mTOR pathway does not contribute to biochemical events that occur as a part of the regulatory mechanisms of cell dynamics [3]. Importantly, TSC1 has been identified as an activator of RhoA and a regulator of cell adhesion. The Rho activating domain of TSC1 overlaps with the region that binds TSC2 indicating that TSC2 may modulate TSC1-dependent activation of RhoA. Our study identified a function of TSC2 as a modulator of the actin cytoskeleton and focal adhesion remodeling. We found that TSC1 inhibits Rac1 and TSC2 blocks this activity of TSC1 which is a prerequisite to the activation of Rac1 and the subsequent inhibition of RhoA; this, in turn, promotes stress fiber disassembly and focal adhesion remodeling. Loss of function of either TSC1 or TSC2 due to inactivating mutations potentially promotes deregulation of the TSC1/TSC2 complex formation followed by deregulation of Rac1 and RhoA activities, which, consequently, induces abnormal cell motility associated with the pathobiology of LAM and TSC.

Funded by NIH HL071106, DOD IDEA TS030012, LAM Foundation, and University of Pennsylvania Research Fund (V.P.K.).

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Abnormal Growth of Smooth Muscle–Like Cells in Lymphangioleiomyomatosis

Role for Tumor Suppressor TSC2

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The TSC1 and TSC2 proteins, which function as a TSC1/TSC2 tumor suppressor complex, are associated with lymphangioleiomyomatosis (LAM), a genetic disorder characterized by the abnormal growth of smooth muscle–like cells in the lungs. The precise molecular mechanisms that modulate LAM cell growth remain unknown. We demonstrate that TSC2 regulates LAM cell growth. Cells dissociated from LAM nodules from the lungs of five different patients with LAM have constitutively activated S6K1, hyperphosphorylated ribosomal protein S6, activated Erk, and increased DNA synthesis compared with normal cells from the same patients. These effects were augmented by PDGF stimulation. Akt activity was unchanged in LAM cells. Rapamycin, a specific S6K1 inhibitor, abolished increased LAM cell growth. The full-length TSC2 was necessary for inhibition of S6 hyperphosphorylation and DNA synthesis in LAM cells, as demonstrated by co-microinjection of the C-terminus, which contains the GTPase activating protein homology domain, and the N-terminus, which binds TSC1. Our data demonstrate that increased LAM cell growth is associated with constitutive S6K1 activation, which is extinguishable by TSC2 expression. Loss of TSC2 GAP activity or disruption of the TSC1/TSC2 complex dysregulates S6K1 activation, which leads to abnormal cell proliferation associated with LAM disease.

Keywords: interstitial lung disease; smooth muscle; TSC

Lymphangioleiomyomatosis (LAM) is a rare lung disorder characterized by the abnormal growth of smooth muscle–like cells within the lung, which promotes cystic destruction of the lung and leads to loss of pulmonary function (1–3). Loss-of-function mutations in the tumor suppressor genes *tuberous sclerosis complex 1* (*TSC1*) and *TSC2* are associated with pulmonary LAM (4–7). The *TSC1* and *TSC2* genes encode two proteins, TSC1 and TSC2, also known as hamartin and tuberin, respectively, which function as a TSC1/TSC2 complex (8). LAM is associated predominantly with *TSC2* mutations, indicating that TSC2 function is critical for sustaining normal cell function (5). Despite considerable research efforts in defining the role of TSC2 in cell proliferation and its relevance to LAM pathobiology, the cellular mechanisms that modulate LAM cell growth remain unknown.

TSC2, a 200-kD, ubiquitously expressed, evolutionary conserved protein, contains in its C-terminus region a GTPase-activating protein (GAP) homology domain that functions as a GAP for the small GTPase Rheb (Ras homologue enriched in brain) (9–12). It has been reported that TSC2 can function as a GAP for small GTPase Rab5, which is critical for vesicular trafficking (13). The functional link between TSC2 and Rab5 remains to be established. TSC2 inhibits Rheb activity by reducing levels of GTP-Rheb, which leads to the suppression of the mammalian target of the rapamycin (mTOR)/S6 kinase 1 (S6K1) signaling pathway (9, 10, 14–17). In the presence of growth factors and abundant nutrients, TSC2 activity is suppressed, leading to increased Rheb and mTOR/S6K1 activity. Studies using established TSC2-deficient cell lines or TSC2 overexpression show that the GAP activity of TSC2 is critical for regulating the mTOR/S6K1 pathway. Rheb overexpression induces mTOR/S6K1 activation in HEK293 cells (10, 15), and TSC2, which has a mutation in the GAP domain, is unable to inhibit Rheb activity and Rheb-dependent activation of S6K1 in COS-7 and HEK293E cells (9, 16). Although Rheb is a physiologic substrate for TSC2 GAP activity, it is not well understood whether the GAP function of TSC2 is necessary and sufficient for inhibiting mTOR/S6K1 activity and cell proliferation in LAM.

TSC2, through its N-terminus region, binds TSC1, and both proteins form the TSC1/TSC2 complex (8, 18–20). Some studies demonstrated that TSC1 and TSC2 are required for maximal GAP activity toward Rheb (9, 11, 16). Other studies have shown that TSC2 alone is sufficient to promote Rheb activation (10, 12). We demonstrated that deletion of the putative TSC1-binding domain of TSC2 attenuates the growth inhibitory effect of TSC2 re-expression in TSC2-deficient ELT3 cells (21). It is poorly understood whether TSC1 contributes to the GAP activity of TSC2 on Rheb.

Although TSC2 may function as a GAP for Rheb, which may contribute to cell proliferation, the role that TSC2 and its GAP domain play in modulating LAM cell growth remains undefined. Using molecular approaches, we show that the C-terminus of TSC2 (amino acids 1114–1784), containing the GAP domain, alone is not sufficient to inhibit ribosomal protein S6 hyperphosphorylation and increased proliferation of LAM cells. Similarly, the N-terminus of TSC2 (amino acids 1–1113), containing the TSC1-binding domain, had little effect on S6 hyperphosphorylation and DNA synthesis. However, co-microinjection of the N-terminus and the C-terminus comprising the full-length TSC2 inhibited the hyperphosphorylation of ribosomal protein S6 and DNA synthesis in LAM cells. Our study indicates that the C-terminus and the N-terminus domain are critical for the negative regulation of mTOR/S6K1 activity and inhibition of LAM cell proliferation.

MATERIALS AND METHODS

Cell Cultures

LAM cells were dissociated from LAM nodules dissected from the lungs of patients with LAM who had undergone lung transplant (22).

(*Received in original form August 3, 2005 and in final form January 10, 2006*)

This work was supported by grants from the LAM Foundation (V.P.K., E.A.G., and D.N.) and NIH grants HL071106 (V.P.K.) and HL67321 (D.N.).

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Am J Respir Cell Mol Biol Vol 34. pp 561–572, 2006
Originally Published in Press as DOI: 10.1165/rcmb.2005-0300OC on January 19, 2006
Internet address: www.atsjournals.org

Briefly, tissue was subjected to an enzymatic digestion in M199 medium containing 0.2 mM $CaCl_2$, 2 mg/ml collagenase D (Roche, Indianapolis, IN), 1 mg/ml trypsin inhibitor (Sigma Chemical Co., St. Louis, MO), and 3 mg/ml elastase (Worthington, Lakewood, NJ). The cell suspension was filtered and washed with equal volumes of cold DF8 medium consisting of equal amounts of Ham's F12 and DMEM supplemented with 1.6×10^{-6} M ferrous sulfate, 1.2×10^{-5} U/ml vasopressin, 1.0×10^{-9} M tri-iodothyronine, 0.025 mg/ml insulin, 1.0×10^{-8} M cholesterol, 2.0×10^{-7} M hydrocortisone, 10 pg/ml transferrin, and 10% FBS. Aliquots of the cell suspension were plated at a density of 1.0×10^4 cells/cm^2 on tissue culture plates coated with Vitrogen (Cohesion Technologies Inc., Palo Alto, CA). The cells were cultured in DF8 medium and were passaged twice a week. LAM cells in subculture during the third through twelfth cell passages were used. LAM-1/1, LAM-2/4, LAM-2/7, LAM-2/8, and LAM-2/9 cells carry *TSC2* gene mutations and have no immunoreactivity to HMB45 (22). The list of LAM cells used in this study is presented in the Table 1. Because there is no established LAM cell nomenclature, we labeled LAM cells in the order in which tissue was acquired from the LAM registry; this corresponds to the first digit in the LAM cell number (Table 1). The second digit corresponds to the cell population derived from the LAM tissue of one patient. For example, the LAM-1/1 represents the total population of cells derived from the LAM nodule of patient number 1; LAM-2/4, LAM-2/7, LAM-2/8, and LAM-2/9 denote cells from the second patient where the tissue was split into different cell populations that were derived and grown separately. In these studies, we used populations 4, 7, 8, and 9. Human bronchus fibroblasts (HBFs), used as a control cells, were dissociated from the bronchus of the same patient with LAM according to the protocol used for LAM cell dissociation. LAM and bronchus tissues were obtained in compliance with the University of Pennsylvania Institutional Review Board approved protocol and the protocol approved by the LAM Registry at the National Heart, Lung, and Blood Institute.

Human airway smooth muscle (ASM) cells, human pulmonary arterial vascular smooth muscle (HVSM) cells, and human lung fibroblasts (HLFs), which were also used as control normal mesenchymal cells, were dissociated and maintained as previously described (22, 23). TSC2-deficient ELT3 and ERC15 cell lines were derived and maintained as previously described (24, 25). All assays were performed on cells maintained in serum-free medium for 48 h.

Transient Transfection and Microinjection

Transient transfection with plasmids, which were prepared using the EndoFree Plasmid Maxi Kit (Qiagen Inc., Valencia, CA), was performed using the Effectene transfection reagent (Qiagen Inc., Valencia, CA) according to the manufacturer's protocol. Microinjection was performed using an Eppendorf Microinjection System (Hamburg, Germany) (22). Briefly, cells were plated on 2-well glass chamber slides (Nalgene Nunc International, Naperville, IL) and transfected or microinjected with pEGFP, pEGFP-N-TSC2, encoding 1–1113 amino acids of TSC2; pEGFP-C-TSC2, encoding 1114–1784 amino acids of TSC2; or co-microinjected with pEGFP-N-TSC2 and pEGFP-C-TSC2 followed by immunocytochemical analysis or 5-bromo-2′deoxyuridine (BrdU) incorporation assay. Immunoblot analysis of GFP-tagged TSC2 constructs was previously described (21).

TABLE 1. LAM CELLS AND HBFs USED FOR EXPERIMENTS

LAM patient number	LAM cells	Normal HBFs from the same patients
1	LAM-1/1	–
2	LAM-2/4	–
	LAM-2/7	
	LAM-2/8	
	LAM-2/9	
3	LAM-3/12	–
4	LAM-4/29	HBF-031202
5	LAM-5/52	HBF-041902

Immunocytochemistry

Cells were fixed with 3.7% paraformaldehyde (Polysciences, Inc., Warrington, PA), permiabilized with 0.1% Triton X-100 (Sigma Chemical Co.), and blocked as previously described (21). Anti-phospho-ribosomal protein S6 (S235) antibody (Upstate Biotechnology, Lake Placid, NY) was used at a 1:50 dilution, anti-GFP rabbit serum (Molecular Probes, Eugene, OR) was used at a 1:200 dilution, and secondary antibody Alexa Fluor 594 donkey anti-sheep IgG conjugate was used at a 1:400 dilution; Alexa Fluor 488 goat anti-rabbit IgG conjugate (Molecular Probes) was used at a 1:400 dilution, and anti-smooth muscle α-actin clone 1A4 FITC-conjugated antibody (Sigma Chemical Co.) was used at a 1:200 dilution. Cells were visualized using a Nikon Eclipse TE2000-E or a Nikon Eclipse E400 microscope (Nikon, Melville, NY) under appropriate filters.

Cell Proliferation Assay

DNA synthesis was assessed by BrdU incorporation assay as described previously (22). Briefly, serum-deprived cells were incubated with BrdU for 24 h followed by immunocytochemical analysis with 2 μg/ml primary mouse anti-BrdU antibody (Becton Dickinson, San Jose, CA) and then with 10 μg/ml secondary Texas Red–conjugated anti-mouse antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). The cells were examined using a fluorescent microscope Nikon Eclipse E400 under ×200 magnification. The mitotic index was defined as the percentage of BrdU-positive nuclei per field per the total number of cells per field. A total of ~ 200 cells were counted per each condition in each experiment.

S6K1 Activity Assay

S6K1 was precipitated from cell lysates with anti-S6K1 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Immunocomplexes were collected by protein A-Sepharose (Pharmacia, Biotech AB, Uppsala, Sweden) followed by an *in vitro* kinase activity assay in the presence of S6K/Rsk2 substrate peptide (KKRNRTLTK) (Upstate Biotechnology) and [γ-^{32}P] ATP (NEN Dupont, Boston, MA). The reaction was stopped by spotting the reaction mixture onto p81 phosphocellulose filters. The radioactivity of samples was measured using a Beckman LS 6500 scintillation counter (22, 26).

Immunoblot Analysis

Serum-deprived cells were lysed in S6K1 or RIPA lysis buffer, and equal amounts of lysate, adjusted to protein content, were subjected to immunoblot analysis with anti-S6K1, anti–phospho-Thr389 S6K1, anti-phospho-Thr421/Ser424 S6K1, anti-ribosomal protein S6, anti–phospho-ribosomal protein S6 (Ser235/236), anti-Akt, anti-phospho-Akt (Ser473), anti-p44/p42 MAP kinase, or anti–phospho-p44/p42 MAP kinase (Thr202/Tyr204) antibodies (Cell Signaling Technology, Inc., Beverly, MA) as previously described (22). Image analysis was performed using the Gel-Pro analyzer program (Media Cybernetics, Silver Spring, MD).

Data Analysis

Data points from individual assays represent the mean values ± SE. Statistically significant differences among groups were assessed with the ANOVA (Bonferroni-Dunn), with values of $P < 0.05$ sufficient to reject the null hypothesis for all analyses. All experiments were designed with matched control conditions within each experiment to enable statistical comparison as paired samples.

RESULTS

Primary LAM Cells Maintain Smooth Muscle α-Actin Expression

Because LAM nodules consist of smooth muscle α-actin–positive cells, we examined whether primary cultures of LAM cells express smooth muscle α-actin in cell culture. LAM cells are spindle-shaped, grow in parallel arrays, and form a distinctive "hills-and-valleys" pattern (Figure 1). Five primary LAM cell cultures derived from the lung of five different LAM patients (*see* Table 1) stained positive for smooth muscle α-actin (Figures 1B–F); these levels were comparable to the levels of smooth muscle α-actin

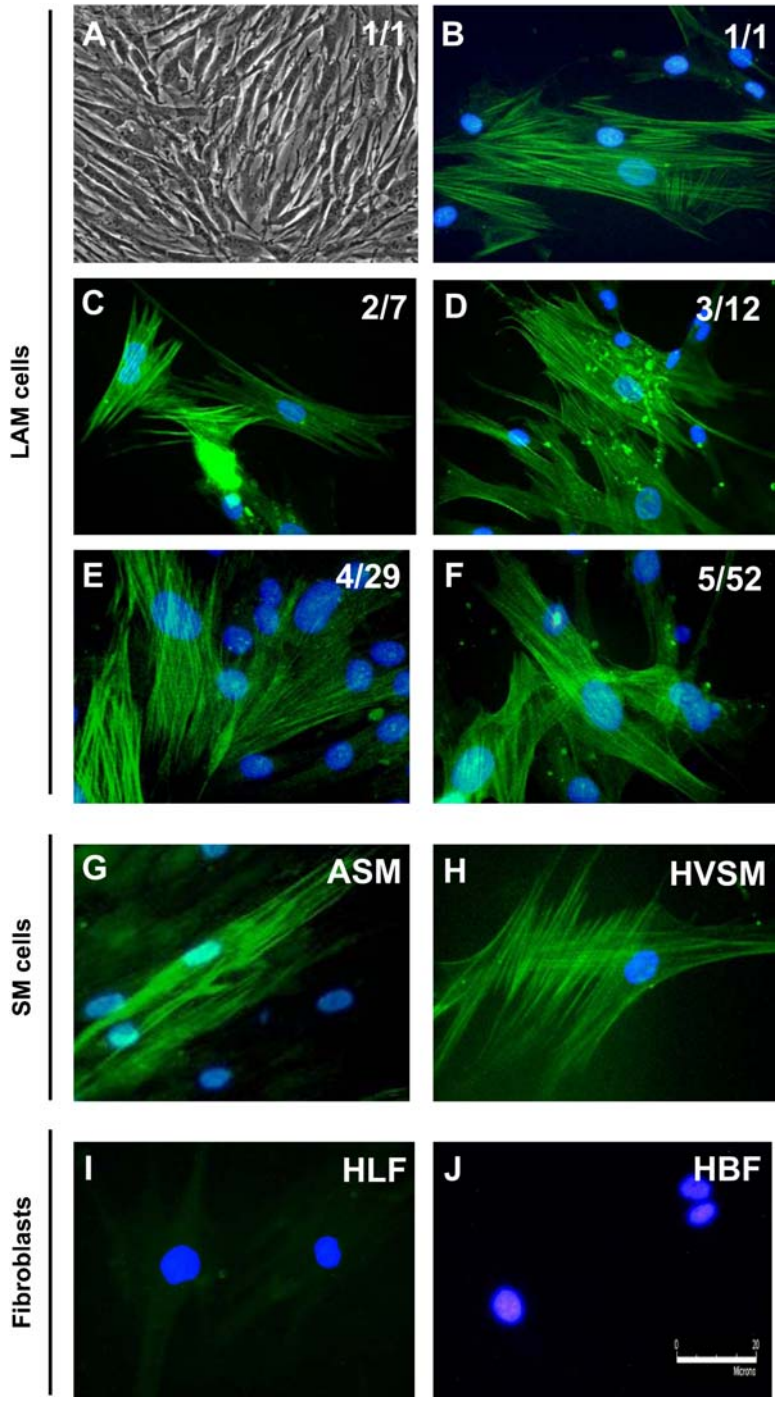


Figure 1. (*A*) LAM cell morphology. LAM cells show typical smooth muscle–like cell morphology. The phase contrast image shown is representative of five LAM cell cultures derived from different patients with LAM. Image was taken on an Olympus IX microscope (*original magnification*: ×200). (*B–J*) Smooth muscle α-actin expression. Serum-deprived LAM-1/1, LAM-2/7, LAM-3/12, LAM-4/29, LAM-5/52, ASM cells, HVSM cells, HLFs, and HBFs were subjected to immunocytochemical analysis with anti-smooth muscle α-actin FITC-conjugated antibody (SM α-actin, *green*) followed by incubation with 4,6-diamidino-2-phenylindole to detect nuclei (*blue*). Images were taken using a Nikon Eclipse TE2000-E microscope (*original magnification*: ×400) and are representative of three separate experiments.

found in primary ASM and HVSM cell cultures (Figures 1G and 1H). In contrast, HBFs, dissociated from the normal bronchus of the same patients with LAM and normal HLFs, were negative for smooth muscle α-actin (Figures 1I and 1J). These data show that primary LAM cells in culture express smooth muscle α-actin, suggesting that these cells retained a smooth-muscle–like phenotype after dissociation from LAM nodules.

LAM Cells Have Increased DNA Synthesis

Because LAM disease is characterized by the abnormal proliferation of smooth muscle-like cells within the lung (1, 2), we examined proliferation levels of LAM cells dissociated from the lung of five different patients with LAM. All five serum-deprived LAM cell cultures had increased DNA synthesis compared with control normal HBFs, HLFs, and HVSM cells (Figure 2A). PDGF stimulation at the concentration that is known to stimulate human smooth muscle cell proliferation (27) further enhanced DNA synthesis in LAM cells compared with diluent-treated cells. PDGF-induced proliferation was markedly higher for all LAM cells compared with control HBFs, HLFs, and HVSM cells, which indicates that growth factors may augment high proliferation rates of LAM cells (Figure 2B). These data show that primary LAM cell cultures have increased DNA synthesis and PDGF further augments LAM cell proliferation.

Because rapamycin, a specific inhibitor of mTOR and a known inhibitor of cell growth (28), has a differential effect on

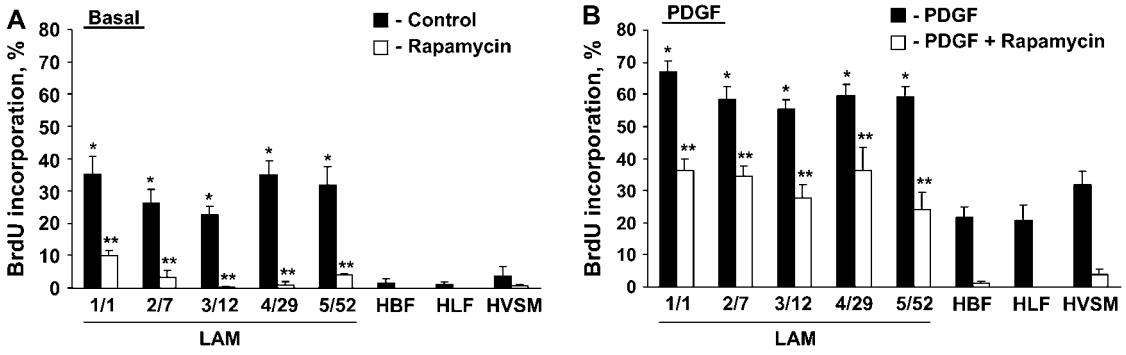


Figure 2. Primary LAM cells have increased DNA synthesis. LAM-1/1, LAM-2/7, LAM-3/12, LAM-4/29, LAM-5/52, HVSM cells, HBFs, and HLFs were serum deprived for 48 h and pretreated for 30 min with 200 nM rapamycin or diluent. Cells were left unstimulated (Basal) (*A*) or were stimulated (*B*) with 10 ng/ml PDGF for 16 h. BrdU was added, and 24 h later, DNA synthesis was assessed by immunohistochemical analysis of BrdU incorporation as described in MATERIALS AND METHODS. BrdU incorporation represents the percentage of BrdU-positive cells compared with the total number of cells. Data represent means ± SE of three separate experiments. *$P < 0.001$ for LAM versus HBFs, HLFs, and HVSM. **$P < 0.001$ for rapamycin-treated cells versus diluent-treated cells by ANOVA (Bonferroni Dunn).

the inhibition of cell proliferation depending on cell type (27, 29–31), we examined whether proliferation of LAM cells derived from different patients is modulated by rapamycin. We have published data that show that rapamycin inhibits LAM-1/1 cell proliferation in a concentration-dependent manner (22). Rapamycin significantly inhibited basal and PDGF-induced DNA synthesis in all analyzed LAM cells (Figures 2A and 2B). Although rapamycin abrogated DNA synthesis of serum-deprived LAM cells, it attenuated only PDGF-induced DNA synthesis, in contrast to the complete inhibition found in HBFs, HLFs, and HVSM cells (Figure 2B). PDGF, a potent mitogen, activates the phosphatidylinositol 3-kinase (PI3K)/S6K1 and ERK pathways (27), which are essential for cell cycle progression and proliferation. Rapamycin abrogates PDGF-induced proliferation in normal HBFs, HLFs, and HVSM cells (Figure 2B). In contrast, in LAM cells the effect of rapamycin is partial, which is ~ 30% inhibition. These data indicate that PDGF may contribute to the differential sensitivity of LAM cells to rapamycin, which might be an important consideration in the therapeutic treatment of LAM with rapamycin.

mTOR/S6K1 Is Constitutively Activated in LAM Cells

Because TSC2 function is associated with the modulation of the mTOR/S6K1 signaling pathway (22, 32, 33) and with the constitutive hyperphosphorylation of ribosomal protein S6, a hallmark of *TSC2* deficiency and loss of TSC2 function, we examined S6 phosphorylation in five primary LAM cells. Immunocytochemical analysis demonstrated an increased phosphorylation of S6 in all five primary LAM cell lines compared with ASM cells (Figure 3A). LAM cells are primary cultures that show some degree of heterogeneity (22). The fraction of phospho-S6–positive cells in the examined LAM cell lines was 76–92% of the total number of cells, depending on cell line; all smooth muscle α-actin–positive LAM cells were also phospho-S6 positive. Only stimulation of ASM cells with PDGF induced S6 phosphorylation comparable with phospho-S6 levels in LAM cells.

Because mTOR is an obligated upstream activator of S6, we examined whether rapamycin affects S6 phosphorylation. ASM cells, stimulated with PDGF, were used as a positive control. Rapamycin inhibited ribosomal protein S6 hyperphosphorylation in serum-deprived LAM-1/1 cells and in PDGF-stimulated ASM cells (Figure 3A). Immunoblot analysis with phospho-S6 antibody further demonstrated that in serum-deprived LAM cells, ribosomal protein S6 was markedly phosphorylated compared with control ASM cells and HBFs (Figure 3B). Treatment with PDGF markedly increased S6 phosphorylation in control HBFs and ASM cells but had little effect on the phospho-S6 level in LAM cells. Quantitative analysis of three separate experiments shows that ribosomal protein S6 is constitutively hyperphosphorylated in serum-deprived LAM cells compared with ASM cells and HBFs, and PDGF significantly promoted S6 phosphorylation in control HBFs and ASM cells but not in LAM cells. The level of PDGF-induced S6 phosphorylation in ASM cells and HBFs was markedly lower than basal S6 phosphorylation in LAM cells (Figure 3C), demonstrating that S6 is hyperphosphorylated in the absence of stimuli in LAM cells. Together, these data show that primary LAM cells have hyperphosphorylated ribosomal protein S6, which is sensitive to rapamycin.

Because S6 phosphorylation is regulated by S6K1, we examined whether S6K1 was activated in primary LAM cell cultures derived from different patients with LAM. Immunoblot analysis of cell lysates revealed that in all LAM cells, S6K1 is phosphorylated on the Thr421/Ser424 and Thr389 residues, which is critical for its activation. In contrast, these sites were not phosphorylated in ASM and HVSM cells that were used as controls (Figure 4A). Analysis of S6K1 activity in the same cells confirmed that primary LAM cell lines have markedly increased S6K1 activity compared with levels detected in ASM and HVSM cells (Figure 4B). These data demonstrate that primary LAM cell cultures are characterized by the constitutive activation of the S6K1/S6 pathway, which is the molecular signature of TSC2 loss of function.

Akt Activity in LAM Cells

Because Akt is a major upstream modulator of the mTOR/S6K1 signaling pathway (34), we examined whether this signaling protein is activated in LAM cells. Analysis of Akt activation was performed with a phospho-Ser-473 Akt antibody, the phosphorylation of which is critical for Akt activation. The immunoblot analysis detected two Akt isoforms in LAM cells and HBFs, with slight differences in phosphorylation levels depending of cell type. However, the total levels of Akt phosphorylation had some, but not marked, differences in serum-deprived LAM cells, ASM cells, and HBFs (Figure 5A, *top panel*). PDGF-stimulated Akt phosphorylation was also comparable in LAM cells, ASM cells, and HBFs (Figure 5A, *middle panel*). These data show that loss of TSC2 function in LAM cells does not affect Akt signaling and that the constitutive activation of mTOR/S6K1 is independent from Akt activity; this correlates with data obtained from other cell types (33).

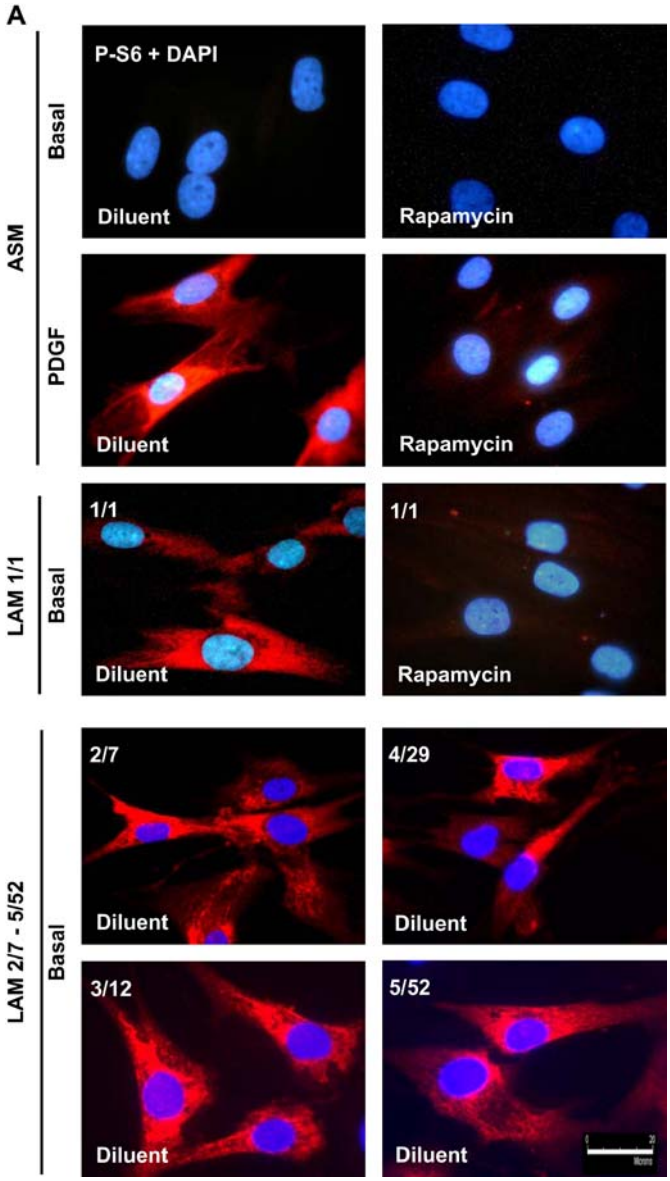


Figure 3. (*A*) Rapamycin inhibits S6 phosphorylation in LAM cells. LAM-1/1, LAM-2/7, LAM-3/12, LAM-4/29, LAM-5/52, and ASM cells were serum deprived, and LAM-1/1 cells were preincubated with 200 nM rapamycin or diluent for 30 min. Control ASM cells were treated with rapamycin followed by stimulation with 10 ng/ml of PDGF. Cells were fixed, and immunocytochemical analysis with anti-phospho-ribosomal protein S6 antibody (P-S6, *red*) was performed followed by incubation with 4,6-diamidino-2-phenylindole (*blue*). Images were taken using a Nikon Eclipse E400 microscope (*original magnification*: ×400) and are representative of three separate experiments. (*B*) Ribosomal protein S6 is hyperphosphorylated in primary LAM cells. Serum-deprived LAM-1/1, LAM-2/7, LAM-3/12, LAM-4/29, LAM-5/52, ASM cells, and HBFs were stimulated with 10 ng/ml PDGF or diluent and subjected to immunoblot analysis with phospho-ribosomal protein S6 (P-S6) and S6 antibodies. Images are representative of three separate experiments. (*C*) Quantitative analysis of S6 phosphorylation. Phosphorylation levels of S6 were calculated using Gel-Pro analyzer software and normalized to total S6 protein levels. P-S6 in ASM cells was taken as a one fold. Data are means ± SE of three separate experiments. *$P < 0.01$ for LAM cells versus ASM cells and HBFs. **$P < 0.01$ for nonstimulated cells versus PDGF-stimulated cells by ANOVA (Bonferroni Dunn).

ERK Is Constitutively Activated in LAM Cells

Because it was shown that ERK can act upstream of TSC1/TSC2 complex and may play a critical role in the phosphorylation and inactivation of TSC2 (1, 35, 36), we examined the activation of ERK in five different LAM cell cultures with phospho-specific antibody. Our data demonstrate that in serum-deprived LAM cells, phosphorylation of ERK is higher compared with ASM cells and HBFs (Figure 5B, *top panel*). PDGF promotes ERK phosphorylation in LAM cells, control ASM cells, and HLFs to comparable levels (Figure 5B, *middle panel*). These data demonstrate that ERK is constitutively phosphorylated in LAM cells in the absence of any stimuli and suggest that ERK-dependent signaling may contribute to LAM disease progression.

Regulation of Ribosomal Protein S6 Phosphorylation Requires the N-Terminus and the C-Terminus of TSC2

TSC2 regulates the Rheb/mTOR/S6K1 signaling pathway through its GAP domain located in its C-terminus (9, 10, 14). Evidence suggests that TSC2 forms a complex with TSC1 through the TSC1-binding domain (1–418 amino acids) located in its N-terminus region (18, 19). We examined whether TSC2 alone is required for modulating mTOR/S6K1 activity or if binding with TSC1 is necessary for TSC2 GAP function. Because ribosomal protein S6 is activated in all LAM cells, we choose the LAM-1/1 cell line for examining this molecular approach. We used DNA constructs encoding GFP-tagged TSC2 regions: the N-terminus (1–1113 amino acids), containing the TSC1-binding domain, and the C-terminus (1114–1784 amino acids), which includes the GAP domain (1517–1674 amino acids) (21). Our data show that only TSC2 constructs containing the TSC1-binding domain N-TSC2 and HBD-TSC2 form a complex and can be co-immunoprecipitated with TSC1 (Figure 6).

LAM cells were microinjected with plasmids, expressing the GFP-tagged, full-length TSC2, N-terminus of TSC2, the C-terminus of TSC2, or control GFP or co-microinjected with the N- and C-terminuses of TSC2 followed by immunocytochemical analysis with anti-phospho S6 antibody. The co-microinjection technique was used to provide direct delivery of equal amounts of C- and N-terminuses TSC2 constructs to the cells. Expression of the N- or the C-terminus of TSC2 alone had little effect on S6

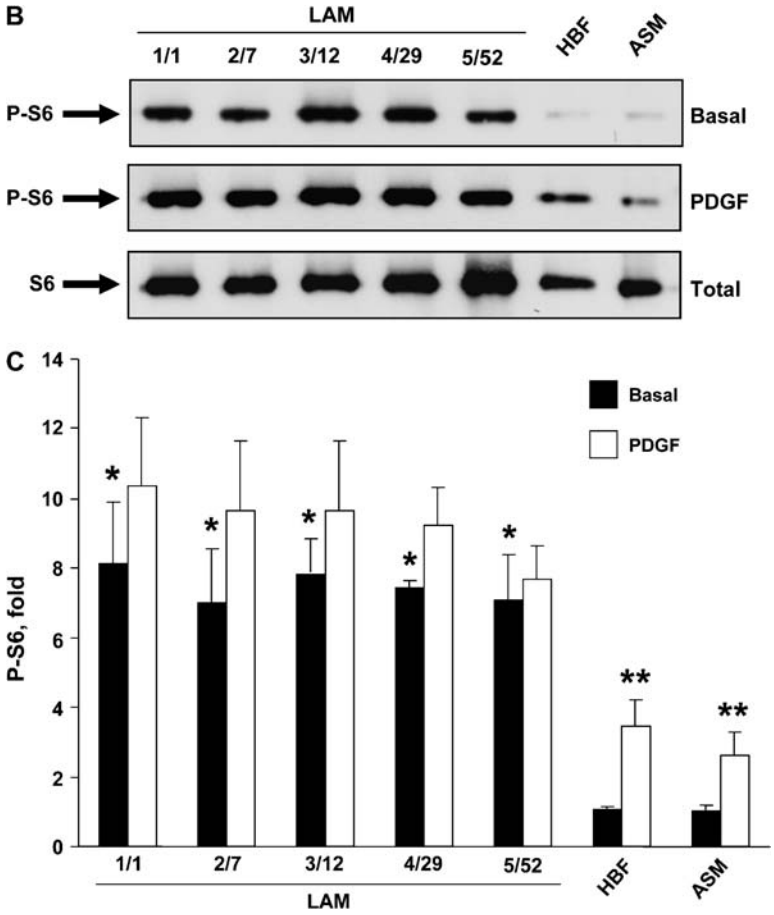


Figure 3. Continued

phosphorylation and was comparable to S6 phosphorylation in cells expressing control GFP (immunofluorescent images are not shown; statistical analysis is presented in Figure 7C). Only co-microinjection of the N- and C-terminuses of TSC2 significantly inhibited S6 phosphorylation (Figure 7A). Similar results were obtained by microinjecting TSC2-deficient ELT3 and ERC15 cells (Figure 7B). Quantitative analysis of the microinjection experiments demonstrates that expression of full-length TSC2 or the C- and N-terminus domains comprising full-length TSC2, but not the C- or N-terminus of TSC2 alone, markedly decreased the phosphorylation level of ribosomal protein S6 in LAM cells (Figure 7C). Similar data were obtained for TSC2-deficient ELT3 and ERC15 cells: Co-injection of N- and C-terminuses of TSC2 decreased S6 phosphorylation by 49.4 ± 2.4% and 56.7 × 3.7%, respectively. These data demonstrate that the C-terminus of TSC2 containing the GAP domain is necessary but not sufficient for regulating S6 phosphorylation and suggest that full-length TSC2 is required for the inhibition of constitutive S6 hyperphosphorylation in LAM cells.

The N- and C-Terminuses of TSC2 Are Required for Inhibition of LAM Cell Proliferation

Because full-length TSC2 is required for inhibition of ribosomal protein S6 hyperphosphorylation, which is critical for cell proliferation, we examined whether these effects correlate with the N- and C-terminus of TSC2 expression on DNA synthesis in LAM cells. LAM, ELT3, and ERC15 cells were microinjected with plasmids expressing the GFP-tagged N-terminus of TSC2, the C-terminus of TSC2, or control GFP or co-microinjected with the N- and C-terminuses of TSC2 followed by BrdU incorporation assay. The N-terminus and C-terminus of TSC2 alone had little effect on LAM cell proliferation, but co-expression of the N- and C-terminals of TSC2 significantly inhibited DNA synthesis by 45.1 ± 6.7%, 49.2 ± 1.2%, and 43.3 ± 3.7% in LAM-1/1, ELT3, and ERC15 cells, respectively (Figure 7D). These data demonstrate that the N- and C-terminals comprising the full-length TSC2 are required for inhibiting LAM and TSC2-deficient cell proliferation.

DISCUSSION

LAM is characterized by the abnormal growth of smooth muscle–like cells within the lung (1–3), which is associated with loss of function of tumor suppressor proteins TSC1 and TSC2 (4–7). In this study, we show that five different primary LAM cell cultures dissociated from the LAM nodules from the lungs of five different patients with LAM have increased DNA synthesis, hyperphosphorylated ribosomal protein S6, and constitutively activated ERK in the absence of any stimuli and that these effects were further augmented by PDGF stimulation. We demonstrate that the C-terminus of TSC2 is necessary but not sufficient for regulating S6 phosphorylation and DNA synthesis in human primary LAM cells. The N-terminus region of TSC2 is also necessary but not sufficient for these functions of TSC2, suggesting that TSC1/TSC2 interaction may be required for the GAP activity of TSC2. Our study indicates that full-length TSC2 and both the C- and N-terminus domains are able to provide optimal regulation of ribosomal protein S6 activation and DNA synthesis in LAM and TSC2-deficient cells.

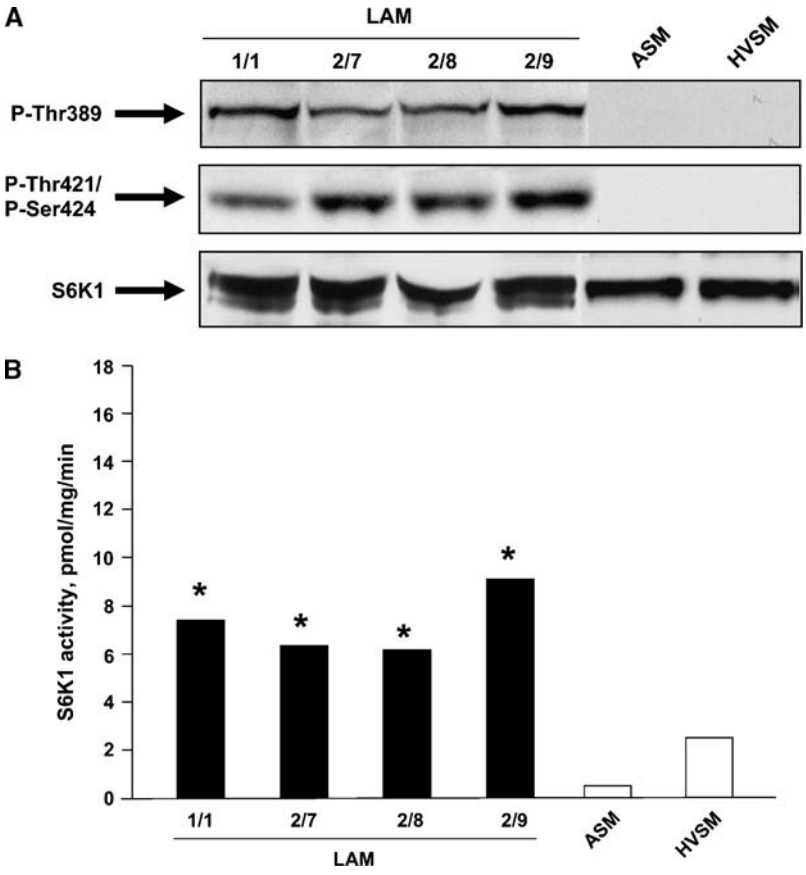


Figure 4. S6K1 is constitutively active in primary LAM cells. (*A*) LAM-1/1, LAM-2/7, LAM-2/8, LAM-2/9, ASM, and HVSM cells were serum deprived, and cell lysates, equal in protein content, were probed with anti–phospho-Thr389 S6K1 (P-Thr389), phospho-Thr421/Ser424 S6K1 (P-Thr421/P-Ser424), or S6K1 antibodies. Images are representative of three separate experiments. (*B*) *In vitro* S6K1 activity was measured in serum-deprived LAM cells, ASM cells, and HVSM cells. $*P < 0.001$ for LAM cells versus ASM and HVSM cells by ANOVA (Bonferroni Dunn).

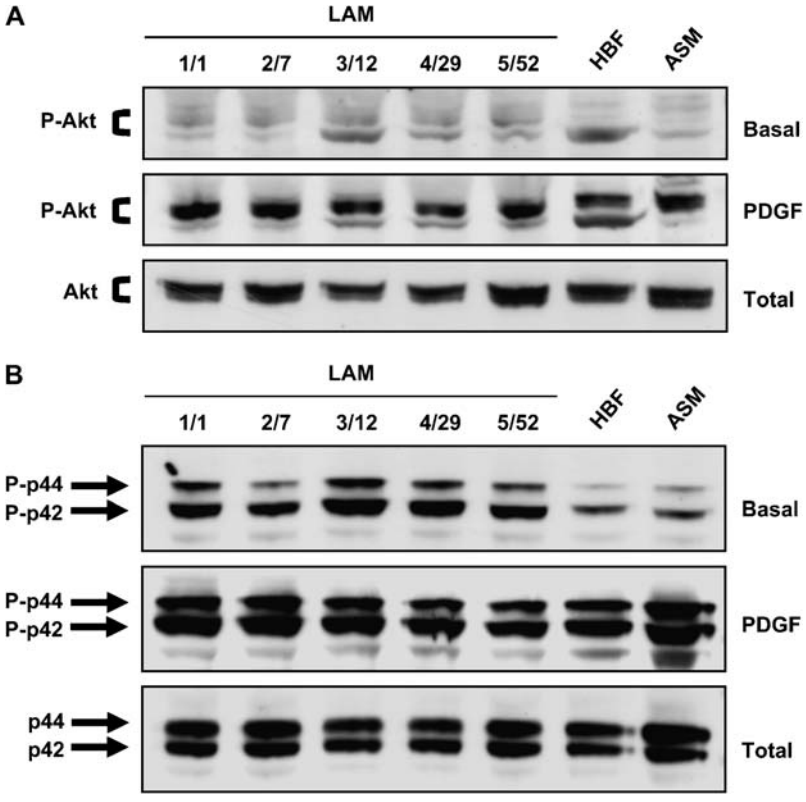


Figure 5. Akt and ERK activity in LAM cells, ASM cells, and HBFs. Serum-deprived LAM-1/1, LAM-2/7, LAM-3/12, LAM-4/29, LAM-5/52 cells, ASM cells, and HBFs were stimulated with 10 ng/ml PDGF or diluent, and immunoblot analysis of cell lysates, equal in protein content, was performed with phospho-Akt (Ser-473) (P-Akt), Akt (*A*), anti-p44/p42 MAP kinase, or anti–phospho-p44/p42 MAP kinase (Thr202/Tyr204) (*B*) antibodies. Images are representative of three independent experiments.

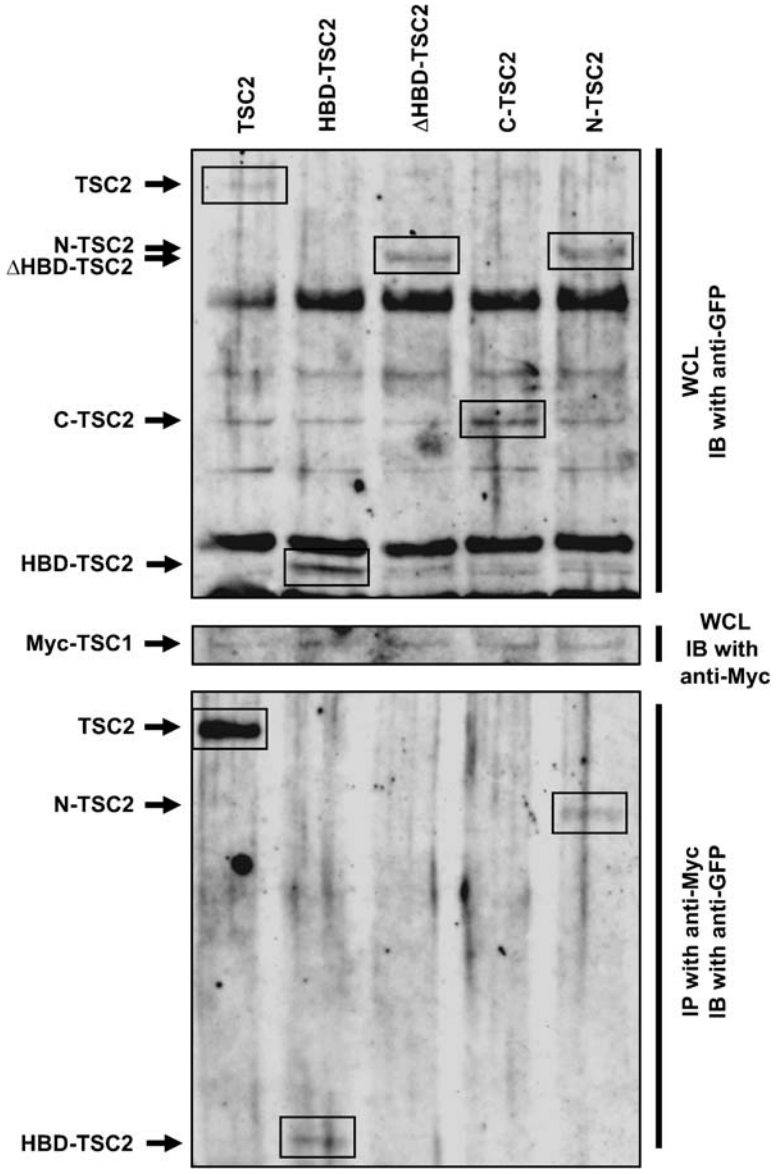
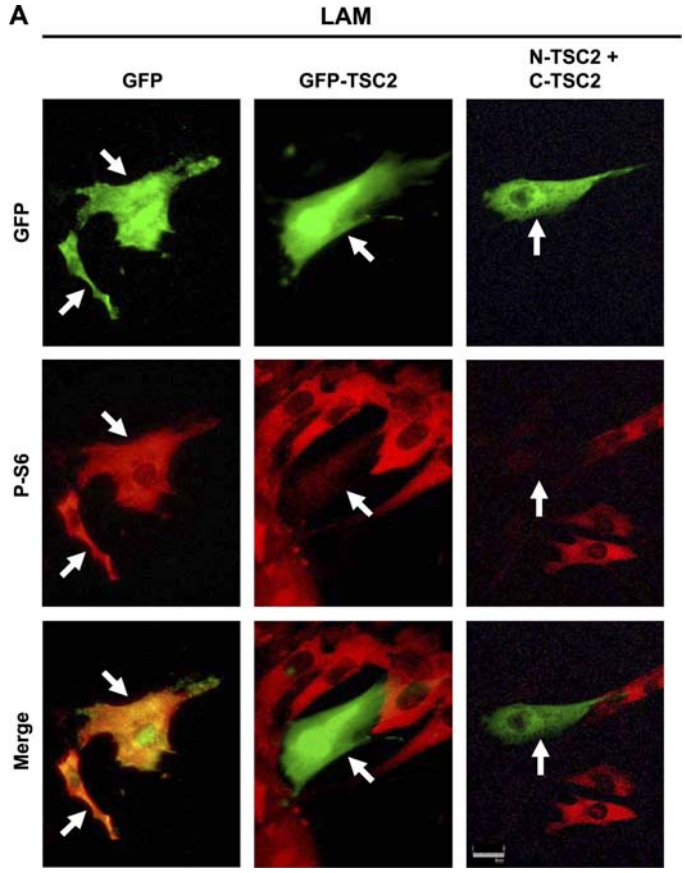


Figure 6. Co-immunoprecipitation of TSC2 constructs with TSC1. Serum-deprived ELT3 cells were co-transfected with Myc-tagged TSC1 and GFP-tagged TSC2 constructs: full-length TSC2, TSC1-binding domain of TSC2 (HBD-TSC2) (1–460 amino acids of TSC2), TSC2 without TSC1-binding domain (ΔHBD-TSC2) (461–1784 amino acids of TSC2), C-TSC2 (1114–1784 amino acids of TSC2), or N-TSC2 (1–1113 amino acids of TSC2). Coexpression of TSC2 constructs and Myc-TSC1 was detected with anti-GFP (*top panel*) and anti-Myc antibodies (*middle panel*), respectively. Immunoprecipitation with anti-Myc antibody was performed, followed by immunoblot analysis of immunoprecipitates with anti-GFP antibody to detect GFP-tagged TSC2 constructs co-precipitated with Myc-tagged TSC1 (*bottom panel*). Images are representative of two separate experiments. *Rectangles* indicate GFP-tagged TSC2 constructs.

LAM can occur as an isolated disorder (sporadic LAM) or in association with TSC, an autosomal disorder affecting 1 in 6,000 individuals who develop hamartomas and benign tumors in the brain, heart, and kidney (37, 38). LAM pathology is characterized by abnormal cell proliferation due to TSC1 and TSC2 loss of function, but disease severity is predominantly associated with the loss of TSC2 (4, 5). Genetic studies of patients with TSC also show that TSC is associated with *TSC1* or *TSC2* mutations (39–42); however, patients with *TSC2* mutations had a greater degree of disease severity compared with those with *TSC1* mutations (40–42). Additionally, loss of TSC2 resulted in abnormal cell proliferation, which was reversed by rapamycin treatment, in TSC2−/− MEFs, ELT3, and ERC15 cells (22, 43). Our data demonstrate that primary LAM cell cultures derived from the lungs of five different patients have increased DNA synthesis without any stimuli, which was inhibited by rapamycin; these results extend our previous findings (22). PDGF further increased the high proliferation rate of LAM cells. The effect of rapamycin on PDGF-stimulated LAM cell proliferation was partial, indicating that mTOR/S6K1-independent mechanisms may be involved in the PDGF-dependent LAM cell proliferation, which might be important for the treatment of LAM disease with rapamycin. Evidence suggests that PDGF activates the PI3K/S6K1 and ERK pathways (27), which are critical for cell proliferation. Our data show that ERK is upregulated in LAM cells and that PDGF further promotes ERK activation. Additionally, inhibition of ERK leads to partial inhibition of PDGF-dependent LAM cell proliferation (our unpublished observation). Taken together, these data suggest that mTOR/S6K1 and ERK signaling pathways may contribute to LAM disease progression, and further studies are needed to establish a role of ERK signaling in PDGF-dependent LAM proliferation. TSC2 suppresses cell growth by regulating mTOR/S6K1 activation (8, 38, 44). Our data show that LAM cell cultures have constitutively active S6K1 and hyperphosphorylated S6 in the absence of any stimuli, which is similar to what was found in TSC2-deficient MEFs, ELT3, and ERC15 cells (22, 43).

TSC2 suppresses cell growth and mTOR/S6K1 activation by functioning as a GTPase-activating protein (10, 38, 44). Recent genetic and biochemical studies identified small GTPase Rheb as a direct target of TSC2 GAP activity (9, 10, 14, 38). However, the function of TSC2 in physiologically relevant cell types, such



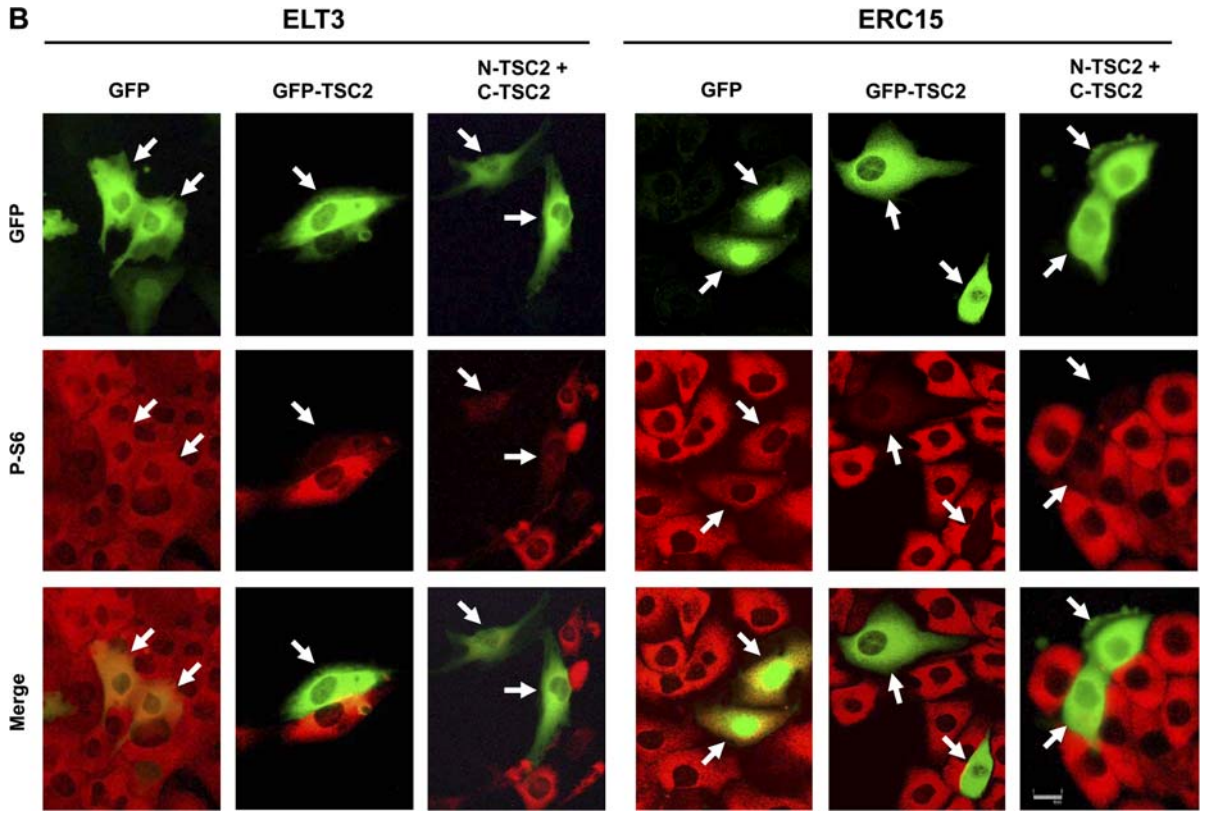


Figure 7. Inhibition of ribosomal protein S6 phosphorylation and cell proliferation require the N- and C-termini of TSC2. Serum-deprived LAM-1/1 (*A*), ELT3, and ERC15 cells (*B*) were microinjected with GFP, GFP-TSC2, or co-microinjected with GFP–N-TSC2 and GFP–C-TSC2 followed by immunostaining with anti-phospho-ribosomal protein S6 (P-S6, *red*) and anti-GFP antibodies (*green*); yellow fluorescence results from the co-localization of P-S6 and GFP in cells. Images were analyzed on a Nikon Eclipse E400 microscope (*original magnification*: ×400) and are representative of three separate experiments. *Arrows* indicate microinjected cells. (*C*) Quantitative analysis of microinjection experiments. Data represent the percentage of P-S6–positive cells per total number of microinjected cells; S6 phosphorylation of GFP-microinjected cells was taken as 100%. Data are means ± SE of three separate experiments. $*P < 0.001$ for GFP-TSC2 or N-TSC2 + C-TSC2 versus GFP by ANOVA (Bonferroni Dunn). (*D*) Serum-deprived LAM-1/1, ELT3, and ERC15 cells were microinjected with plasmids expressing GFP as a control, GFP–N-TSC2, or GFP–C-TSC2 or co-microinjected with GFP–N-TSC2 and GFP–C-TSC2 followed by BrdU incorporation assay. BrdU incorporation represents the percentage of BrdU-positive cells expressing GFP-tagged constructs compared with the total number of transfected cells. BrdU incorporation of GFP-microinjected cells was taken as 100%. Data represent means ± SE of three separate experiments. $*P < 0.001$ for N-TSC2 + C-TSC2 versus GFP by ANOVA (Bonferroni Dunn).

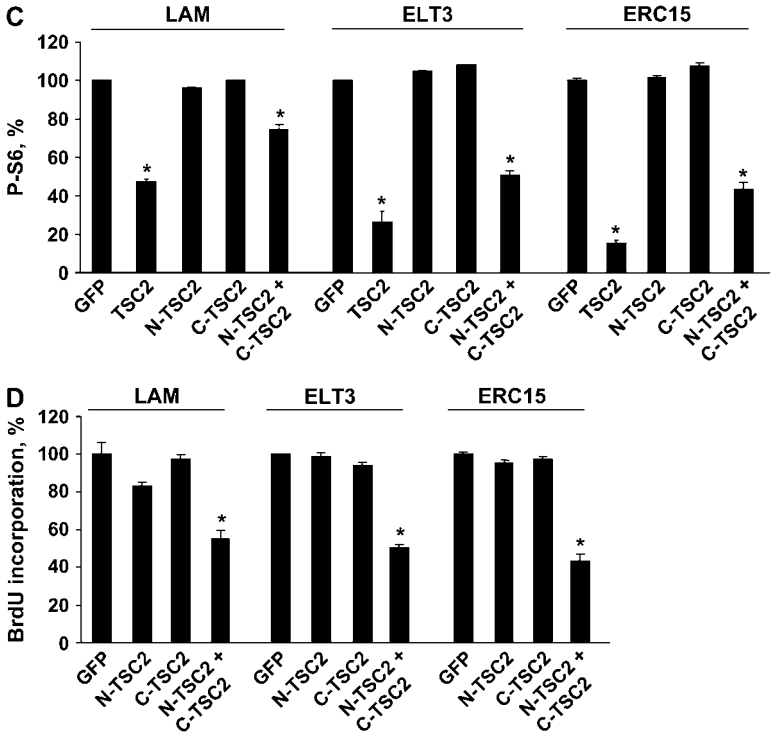


Figure 7. Continued

as human LAM cells, remains to be determined. In our study, we show that the C-terminus of TSC2, amino acids 1114–1784, is necessary but not sufficient for inhibiting ribosomal protein S6 phosphorylation and DNA synthesis. The stable expression of the larger truncated mutant of the C-terminus of TSC2, amino acids 1049–1809, in Eker tumor cell lines LExF2 and ERC18M partially decreased colony formation and showed partial morphologic reversion compared with full-length TSC2 (45). TSC2 constructs coding the C-terminus region, including the GAP domain, exhibited suppressed tumorigenicity in the Eker rat (45, 46). Our published studies also demonstrate that expression of the larger C-terminus TSC2 mutant partially inhibits S6 phosphorylation and DNA synthesis (21). Collectively, these data indicate that other regions containing, for example, Akt, AMPK, or MAPK phosphorylation sites, are required for the GAP function of TSC2.

Evidence suggests that Akt functions as an upstream modulator of growth factor-induced mTOR/S6K1 activation (34). Akt through phosphorylation of TSC2 releases mTOR/S6K1 from TSC2-dependent inhibition (33). Our data show that in primary LAM cell cultures, Akt is not activated in the absence of any stimuli and that PDGF-stimulated Akt phosphorylation is comparable to levels found in ASM cells and HBFs. These data correlate with our published studies demonstrating that basal levels of Akt phosphorylation were comparable in LAM, TSC2−/− ELT3, and ASM cells (22) and that PDGF promoted a marked activation of PI3K in the TSC2−/− ELT3 cells (47). Additionally, it was shown that loss of TSC2 in TSC2−/− MEFs inhibits activation of Akt by IRS-dependent growth factor pathways, such us insulin or IGF-I, but has little effect on IRS-independent PDGF-induced Akt activation in TSC2−/− MEFs (48). In contrast, Zhang and colleagues demonstrated that PDGF receptor (PDGFR) α and PDGFR β are downregulated in the TSC2−/− MEFs, which led to a reduction of PI3K/Akt activation by PDGF, insulin, and serum (43). We found that PDGFR β is expressed in TSC2−/− rat ELT3 cells and is activated upon PDGF stimulation; furthermore, the re-expression of TSC2 in these cells had little effect on PDGFR β levels, suggesting that PDGF-induced signaling is not defective at the receptor level (21).

TSC2 functions in a complex with TSC1, the loss of which leads to mTOR/S6K1 activation and cell growth (39). TSC2 binds TSC1 through its specific binding domain located in its N-terminus region (18, 49, 50); the N-terminus of TSC2 and TSC1 have no GAP activity toward Rheb (10–12). However, a number of studies on mammalian cell lines indicated that TSC1/TSC2 interaction is required for TSC2 function as a regulator of the Rheb/mTOR/S6K1 signaling pathway (9–11, 16). Inoki and colleagues demonstrated that the GAP activity of TSC2 requires the full-length protein because neither the N-terminus nor the C-terminus region, which contains the GAP domain, displayed GAP activity toward Rheb in HEK293 cells (10). Tee and colleagues showed that overexpression of TSC2 or TSC1 in HEK293 cells enhanced the intrinsic GTPase activity of Rheb by $\sim$ 2-fold, but co-expression of TSC2 and TSC1 enhanced Rheb GTPase activity by $>$ 100-fold over the activity of either alone (16). Garami and colleagues reported that TSC1 alone had no effect on the amount of GTP-bound Rheb but found strongly potentiated GAP activity of TSC2 in COS cells (9). Nellist and colleagues demonstrated that some missense mutations outside the TSC2 GAP domain completely inactivated the TSC1/TSC2 complex, inhibited TSC2 GAP activity, and promoted the phosphorylation of the downstream effectors of mTOR in MEFs (20). Because the binding of TSC1 with TSC2 prevents TSC2 ubiquitination and degradation (50, 51), it is possible that TSC1 promotes the GAP function of TSC2 by its stabilization. Our data show that the N-terminus region of TSC2 alone has little effect on S6 phosphorylation and DNA synthesis, but its presence is necessary for the inhibition of S6 phosphorylation and DNA synthesis in human LAM and TSC2-deficient ELT3 and ERC15 cells. Taken together, these data indicate the

importance of TSC1/TSC2 interaction for the function of TSC2 as a negative regulator of the Rheb/mTOR/S6K1 pathway. Our data also demonstrate that the GAP function of TSC2 is necessary for the inhibition of cell proliferation: The N-terminus of TSC2 without the GAP domain is unable to inhibit DNA synthesis in human LAM and TSC2-deficient rat cells; however, co-microinjection of the N- and C-terminuses comprising full-length TSC2 inhibits LAM cell proliferation.

In summary, our current findings provide evidence that the C-terminus of TSC2 is necessary, but not sufficient, for regulating S6 activity and DNA synthesis in primary human LAM cells and in TSC2-deficient ELT3 and ERC15 cells. The C- and N-fragments, containing the GAP domain and the TSC1-binding domain, respectively, are involved in ribosomal protein S6 and DNA synthesis inhibition, suggesting that the GAP activity of TSC2 and TSC1/TSC2 interaction are required for TSC2 to function as a tumor suppressor. Because the loss of TSC2 or TSC1 functions is associated with LAM disease (4–7), the loss of TSC2 GAP activity or the loss of the ability TSC2 and TSC1 to form a complex may de-regulate GAP-dependent regulation of the Rheb/mTOR/S6K1 signaling pathway and LAM cell growth, which could be key events in the pathobiology of LAM.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Acknowledgments: The authors thank Dr. Reynold A. Panettieri, Jr., for a gift of ASM cells, Dr. Cheryl L. Walker for ELT3 and ERC15 cell lines, and LAM patients and the LAM Registry at National Heart, Lung and Blood Institute for providing LAM and bronchus tissue.

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Modulation of Cell Migration and Invasiveness by Tumor Suppressor TSC2 in Lymphangioleiomyomatosis

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The loss of TSC2 function is associated with the pathobiology of lymphangioleiomyomatosis (LAM), which is characterized by the abnormal proliferation, migration, and differentiation of smooth muscle–like cells within the lungs. Although the etiology of LAM remains unknown, clinical and genetic evidence provides support for the neoplastic nature of LAM. The goal of this study was to determine the role of tumor suppressor TSC2 in the neoplastic potential of LAM cells. We show that primary cultures of human LAM cells exhibit increased migratory activity and invasiveness, which is abolished by TSC2 re-expression. We found that TSC2 also inhibits cell migration through its N-terminus, independent of its GTPase-activating protein activity. LAM cells show increased stress fiber and focal adhesion formation, which is attenuated by TSC2 re-expression. The small GTPase RhoA is activated in LAM cells compared with normal human mesenchymal cells. Pharmacologic inhibition of Rho activity abrogates LAM cell migration; RhoA activity was also abolished by TSC2 re-expression or TSC1 knockdown with specific siRNA. These data demonstrate that TSC2 controls cell migration through its N-terminus by associating with TSC1 and regulating RhoA activity, suggesting that TSC2 may play a critical role in modulating cell migration and invasiveness, which contributes to the pathobiology of LAM.

Keywords: lung; RhoA GTPase; smooth muscle cells; TSC1

Lymphangioleiomyomatosis (LAM) is a rare lung disease affecting primarily women of childbearing age; it is characterized by the abnormal and potentially neoplastic growth of smooth muscle–like cells within lungs, which leads to the cystic destruction of the lung interstitium and the loss of pulmonary function (1–3). LAM can be sporadic or manifested in association with tuberous sclerosis complex (TSC), an autosomal dominant inherited disorder affecting 1 in 6,000 individuals who develop hamartomas and benign tumors in the brain, heart, and kidneys; it is also manifested by cognitive defects, epilepsy, and autism (4, 5). Importantly, malignant tumors of the kidney, which develop either as malignant angiomyolipomas or renal cell carcinomas, occur in 50% of sporadic LAM (6) and in most patients with TSC (7). Circulating neoplastic smooth muscle–positive LAM cells show loss of heterozygosity (LOH) for *TSC2* (8). To date, however, little information is available concerning whether the tumor suppressor protein TSC2 contributes to neoplastic LAM cell dissemination.

TSC2 forms a heterodimer with tumor suppressor TSC1 and functions as a TSC1/TSC2 protein complex (9, 10). TSC1 regulates the activity of RhoA GTPase and associates with the cortical ezrin/radixin/moezin (ERM) proteins, which serve as molecular bridges between the plasma membrane and cortical actin filaments (11). Evidence that TSC2 binds TSC1 through the TSC1-binding domain located in the C-terminus of TSC2 (12–14), which overlaps with the Rho-activating domain of TSC1 (11), suggests that TSC1/TSC2 complex formation may be involved in the regulation of RhoA GTPase activity and, ultimately, cell migration. TSC2 functions as a modulator of the actin cytoskeleton and focal adhesion remodeling (15). TSC1 inhibits Rac1 and TSC2 blocks this activity of TSC1, which is a prerequisite for the activation of Rac1 and the subsequent inhibition of RhoA; this, in turn, promotes stress fiber disassembly and focal adhesion remodeling (15). Loss of function of either TSC1 or TSC2 due to inactivating mutations potentially promotes the deregulation of the TSC1/TSC2 complex formation followed by deregulation of Rac1 and RhoA activities, which, consequently, induces abnormal cell motility associated with the pathobiology of LAM.

Until recently LAM and TSC tumors were considered benign. Compelling evidence, however, supports a neoplastic model for LAM, suggesting a link between loss of TSC1/TSC2 function and cell invasion and metastasis (16). Identical *TSC2* mutations and LOH patterns were detected in pulmonary LAM and in angiomyolipoma cells from the same patients with LAM with renal angiomyolipoma, suggesting that these cells have a common origin (17). Secondary tumor formations with the same *TSC2* mutations were found in lymph nodes of patients with sporadic LAM (18). In addition, identical *TSC2* mutations were found in native LAM cells and recurrent LAM cells after lung transplantation (19). The presence of the same *TSC2* mutation in pulmonary LAM, LAM cells in the lymph nodes, and recurrent LAM after transplant strongly supports the notion that LAM cells migrate abnormally and metastasize *in vivo*. This was further supported by a recent report establishing the existence of disseminated, potentially metastatic LAM cells with *TSC2* LOH in bodily fluids of patients with LAM (8). Thus, clinical and genetic evidence suggest that TSC2 loss of function is associated with neoplastic cell growth in LAM. However, the cellular and molecular mechanisms of these effects have not been established.

The goal of this study was to examine LAM cell migration and invasiveness, and to establish whether a link exists between TSC2 function and cell motility. We found that LAM cells from the lung tumors of patients with LAM show invasiveness, increased rate of migration, and RhoA activation; expression of TSC2 reverses these effects. The downregulation of TSC1 levels also inhibits RhoA activation; and the N-terminus of TSC2 is necessary and sufficient for inhibition of LAM cell migration, indicating that TSC1 and TSC2 are involved in the regulation of LAM cell migration.

MATERIALS AND METHODS

LAM Cell Culture

We used primary cultures of LAM-derived (LAM) smooth muscle–like cells dissociated from the LAM nodules of five patients who had

(*Received in original form October 5, 2005 and in final form December 20, 2005*)

This work was supported by grants from the NIH HL071106 (to V.P.K.), DOD IDEA TS030012 (to V.P.K.), ALA CI-9813-N (to V.P.K.), NIH HL67321 (to D.N.), and The LAM Foundation (to V.P.K, D.N., and E.A.G.).

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Am J Respir Cell Mol Biol Vol 34. pp 473–480, 2006
Originally Published in Press as DOI: 10.1165/rcmb.2005-0374OC on December 30, 2005
Internet address: www.atsjournals.org

undergone a lung transplant as we previously described (15, 20); all tissues were acquired in accordance with protocols approved by the University of Pennsylvania Institutional Review Board. Briefly, cells were dissociated by enzymatic digestion, plated on Vitrogen-coated plates (Cohesion Technologies Inc., Palo Alto, CA), and maintained in DF8 medium (equal amounts of Ham's F12 and Dulbecco's modified Eagle's medium with 1.6×10^{-6} M ferrous sulfate, 1.2×10^{-5} U/ml vasopressin, 1.0×10^{-9} M triiodothyronine, 0.025 mg/ml insulin, 1.0×10^{-8} M cholesterol, 2.0×10^{-7} M hydrocortisone, and 10 pg/ml transferrin) supplemented with 10% FBS. Before all experiments, cells were serum deprived for 24 h. In culture, LAM cells exhibited smooth muscle–like cell appearance: long spindle-shaped cells growing in parallel arrays that form a "hills-and-valleys" pattern. LAM cells from each patient with LAM were characterized on the basis of smooth muscle (SM) α-actin expression, S6K1 activity, ribosomal protein S6 phosphorylation, and DNA synthesis. All LAM cells used in this study had constitutively activated S6K1, hyperphosphorylated ribosomal protein S6, and a high degree of proliferative activity in the absence of any stimuli as well as a filamentous expression pattern of smooth muscle α-actin (20, 21). Mutational analysis of the *TSC2* gene was performed in LAM cells from two patients with LAM and was reported in our previously published study (20). As controls, we used primary cultures of normal human bronchus fibroblasts (HBFs) derived from the bronchus of the patients with LAM; two matched HBFs and LAM cells from the same patients were used in this study. In contrast to LAM cells, HBFs had low basal S6K1 activity and DNA synthesis (data not shown). As an additional control we used human airway smooth muscle (ASM) cells. ASM cells were dissociated from human trachea, which was obtained from human lung transplant donors and have been previously described (20, 22). We recognize that LAM cells have some limitations and show some degree of heterogeneity; for example, hyperphosphorylated ribosomal protein S6 is found in ~ 80% LAM cells. HBFs and ASM cells may also be not the best controls for LAM cells; however, HBFs and ASM cells may be reasonable controls for LAM cells, given that currently there is no perfect LAM cell model. All experiments were performed with a minimum of three different HBF and ASM cell lines.

Cell Migration Assay

Cell motility was examined using a Boyden chamber apparatus as we described previously (23–25). Serum-deprived LAM, HBF, and ASM cells were briefly trypsinized by 0.05% trypsin/0.53 mM EDTA, centrifuged at 900 rpm for 10 min, and resuspended in serum-free media supplemented with BSA. Cells (5×10^4) were then placed into the upper wells of the Boyden chamber fitted with an 8-μm pore membrane coated with Vitrogen (100 μg/ml). Agonists or vehicle in serum-free media supplemented with BSA were added to the lower chambers. Cells in the Boyden chamber were incubated for 4 h at 37°C in a 5% CO_2 incubator. Nonmigrated cells were scraped off; the membrane was fixed with methanol, stained with Hemacolor stain set (EM Industries, Inc., Gibbstown, NJ), and scanned. Cell migration was analyzed using the Gel-Pro Analyzer program (Media Cybernetics, Silver Spring, MD).

Collagen Invasion Assay

Confluent cells were trypsinized, resuspended in media with or without growth factors, and then 1×10^5 cells were placed in each well of a 6-well plate coated with collagen type I gel (Upstate Cell Signaling Solutions, Lake Placid, NY) as described previously (26). After 24 h the number of single individual cells that migrated into the collagen gel deeper than 10 μM (deep cells), and the number of superficial cells, were counted using a Nikon Eclipse TE2000-E inverted microscope (Nikon, Melville, NY) and Image-Pro Plus software (Media Cybernetics, Silver Spring, MD). The invasion index was calculated according to the following formula: (deep cells) × 100/(deep + superficial cells), and is expressed as a percentage.

Transient Transfection and Immunostaining

Plasmids were prepared using the EndoFree Plasmid Maxi Kit (Qiagen Inc., Valencia, CA). Transient transfection was performed using the Effectene transfection reagent (Qiagen) according to the manufacturer's protocol. Twenty-four hours after transfection, transfection efficiency was assessed and expressed as a percentage of transfected cells per total number of cells; then the cell migration assay was performed. The membrane with migrated cells was fixed with 3.7% paraformaldehyde (Polysciences, Inc., Warrington, PA) for 15 min, treated with 0.1% Triton X-100 (Sigma, St. Louis, MO) for 20 min at room temperature, and then blocked with BSA in 20 mM TRIS (pH 7.5)–150 mM NaCl (TBS) for 1 h at 37°C. After incubation with primary anti-GFP rabbit serum (Molecular Probes, Eugene, OR), 1:200 dilution, and secondary Alexa Fluor 488 Goat anti-rabbit IgG conjugate (Molecular Probes), 1:400 dilution antibodies for 1 h at 37°C, respectively, the membrane was mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA). The cells were visualized on a Nikon Eclipse E400 microscope under ×200 magnification. Cell migration was defined as the percentage of transfected cells migrated through the membrane compared with the total number of transfected cells plated on membrane.

Rho Activation Assay

Rho activity was measured using a Rho Activation Assay Kit (Upstate Cell Signaling Solutions) according to the manufacturer's protocol. Cells were lysed in MLB buffer, then incubated with Rhotekin RBD-conjugated agarose for 1 h; beads were then collected, washed, resuspended in 2× Laemmli reducing sample buffer, boiled, and SDS-polyacrylamide gel electrophoresis and immunoblotting analysis were performed. Rho was detected using anti-Rho (A, B, C) antibody.

Data Analysis

Statistical analysis of F-actin staining was performed by visual and quantitative analysis of digital images taken using a Nikon Eclipse E400 microscope at ×200 magnification using Gel-Pro Analyzer software (Media Cybernetics) (15). Data points from individual assays represent the mean values ± SE. Statistically significant differences among groups were assessed with the ANOVA (Bonferroni-Dunn), with values of $P < 0.05$ sufficient to reject the null hypothesis for all analyses. All

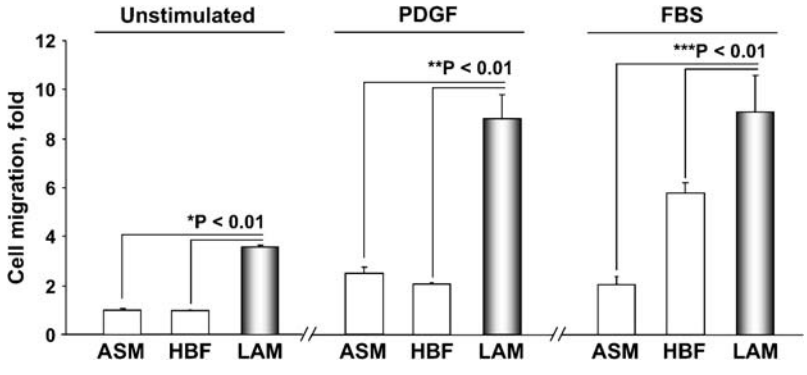

Figure 1. LAM cell migration. Serum-deprived LAM, ASM cells, and HBFs were placed on collagen-saturated membranes in serum-free medium, and allowed to migrate in the Boyden chamber for 4 h in either the absence of any stimuli, in the presence of 10 ng/ml PDGF-BB, or 10% fetal bovine serum (FBS); membranes were then fixed, stained with Hemacolor stain set, and analyzed using Gel-Pro software (Media Cybernetics). Migration was assessed as the number of cells invaded into the membrane after 4 h of incubation. Migration of ASM cells without stimuli was taken as 1-fold, which was equal to 289.7 ± 16.4 cells per field. Data represent mean values ± SE from measurements performed in triplicate from three independent experiments using LAM cells, HBF, and ASM cells from different patients in each experiment by ANOVA (Bonferroni-Dunn).

experiments were designed with matched control conditions within each experiment to enable statistical comparison as paired samples.

RESULTS

Increased Migratory Activity and Invasiveness of LAM Cells

Abnormal cell migration and invasiveness are among the characteristic features of tumors, which may occur, in part, due to the deregulation of TSC2 signaling, and could be augmented by promigratory growth factors, such as PDGF and serum containing the critical promigratory factor lysophosphatidic acid (LPA) (11, 23, 25, 27). Since the difference between normal and tumor cells lies in their ability to migrate continuously and invade extracellular matrixes (28–31), we examined the migration and invasiveness of LAM cells. The LAM cells used in this study had constitutively activated S6K1 and hyperphosphorylated ribosomal protein S6, which are molecular signatures of TSC2 dysfunction, and increased DNA synthesis, compared with normal HBF and ASM cells used as control cells (data not shown). Migration of LAM cells was examined using the Boyden chamber migration assay. The serum-deprived LAM, ASM cells, and HBFs were plated on the collagen-saturated membrane of the Boyden chamber in the absence of any stimuli, and were allowed to migrate for 4 h. Unstimulated LAM cells had significantly higher migratory activity compared with the chemokinesis of HBFs and ASM cells (Figure 1). Importantly, when LAM cells were stimulated with either PDGF or FBS, their migratory activity was further increased compared with both unstimulated LAM cells, ASM cells, and HBFs (Figure 1), indicating that promigratory growth factors enhance LAM cell migration.

Next we examined the ability of LAM cells to invade a collagen matrix. As seen Figure 2A, LAM cells invaded the collagen and after 24 h were found at the 20-μm depth in the collagen. In contrast, human ASM cells were found only in the top collagen layer, which remained unchanged for 48 h and 72 h (data not shown). Similar experiments were performed using HBFs, which are summarized in Figure 2B: 40.5 ± 7.3% LAM cells invaded collagen matrix; in contrast, only 3.9 ± 2.3% HBF and 3.6 ± 3.6% ASM cells were found in the collagen layer. Collectively, our data show that primary human LAM cells, which carry TSC2 gene mutations but retain TSC1 expression (15, 20), have increased migratory activity and invade the collagen matrix, which may be critical in the neoplastic cell dissemination in LAM disease.

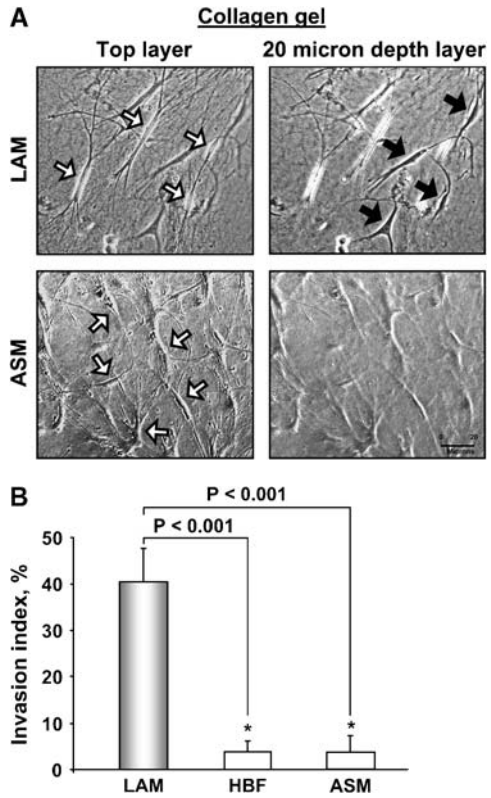

Figure 2. LAM cell invasiveness. (*A*) LAM or ASM cells were plated on 6-well plates coated with collagen type I. After 24 h, cell invasion was analyzed using a Nikon Eclipse TE2000-E inverted microscope and Image-Pro Plus software. The collagen gel was traversed from top to bottom, and images were taken in 5-μm steps. Images are representative of three independent experiments. *White arrows* indicate cells located at the top collagen layer; *black arrows* indicate cells located at the 20-μm depth of the collagen layer. The quantity of invaded ASM cells did not increase after 48 h and 72 h of incubation (data not shown). (*B*) Statistical analysis of the invasion assay of LAM, ASM cells, or HBFs. Single individual cells found deeper than 10 μm were interpreted as cells migrated into the gel, or "deep" cells. The invasion index was calculated as the percentage of "deep" cells per total number of cells taken as 100%. Eight to ten fields containing 80–120 cells per field were analyzed for each cell type. Data represent mean values ± SE from three independent experiments by ANOVA (Bonferroni-Dunn).

Expression of TSC2 Inhibits the Increased Migratory Activity and Invasiveness of LAM Cells

To determine whether LAM cell invasiveness is associated with TSC2 dysfunction, we examined whether TSC2 expression is sufficient for the inhibition of LAM cell invasiveness. As shown in Figure 3, transfection of LAM cells with TSC2 significantly inhibited invasiveness, indicating that TSC2 may be involved in regulating LAM cell invasiveness.

To define whether TSC2 is required for the regulation of cell migration, we examined whether re-expression of TSC2 will affect increased LAM cell migration. Expression of TSC2 significantly inhibited LAM cell migration compared with cells transfected with control GFP plasmid. As seen in Figure 4, most of the LAM cells transfected with GFP (99.7 ± 9.2%) maintained invasive motility in the absence of any stimuli. In contrast, only 37.1 ± 5.1% of the total number of cells expressing GFP-tagged TSC2 migrated into the collagen-saturated matrix in serum-free medium. These data demonstrate that TSC2 may regulate cell migration, and supports our hypothesis that loss or mutation of TSC2 may promote abnormal cell motility in LAM.

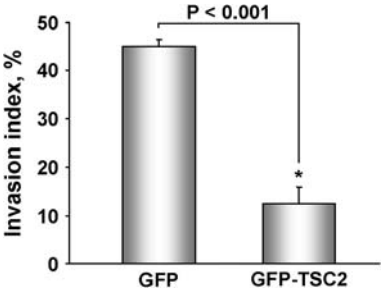

Figure 3. TSC2 inhibits LAM cell invasiveness. Cells were transfected with GFP-TSC2 or GFP as a control followed by the cell invasion assay. The invasion index was calculated as the percentage of GFP-positive "deep" cells, found deeper than 10 μm, per total number of GFP-positive cells taken as 100%. Data represent mean values ± SE from two independent experiments by ANOVA (Bonferroni-Dunn).

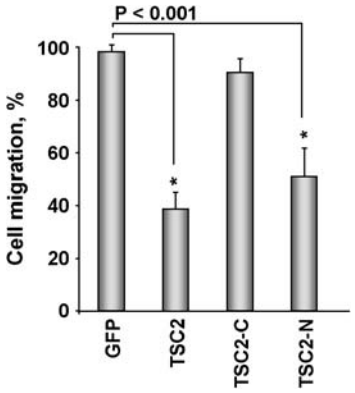

Figure 4. TSC2 inhibits LAM cell migration, and the N-terminus (but not the C-terminus) of TSC2 is sufficient for this inhibition. Quantitative analysis of the migration assays of LAM cells transfected with GFP, GFP-TSC2, GFP-C-terminus TSC2 (TSC2-C), or GFP-N-terminus TSC2 (TSC2-N). Cells were transiently transfected with plasmids; cell transfection efficiency was then assessed, and transfected cells were allowed to migrate in a Boyden chamber for 4 h; membranes were fixed, then immunostained with anti-GFP antibody to detect GFP-tagged TSC2 constructs and GFP. Data represent the percentage of migrated transfected cells per total number of transfected cells plated on the membrane. Data represent mean values ± SE from two experiments. *$P < 0.001$ for TSC2-N or TSC2 versus GFP by ANOVA (Bonferroni-Dunn).

To further address the mechanism of invasive LAM cell motility, we next examined which structure–functional domain of TSC2 is important for inhibiting LAM cell migration. We found that the N-terminus of TSC2 was sufficient, and as effective, in inhibiting cell migration as full-length TSC2 (Figure 4). Importantly, the C-terminus, which contains the GAP domain that is involved in regulating TSC2-dependent protein translational regulation and cell proliferation (32), has little effect on cell migration. Our data demonstrate the functional role of the N-terminal of TSC2 in regulating cell migration.

RhoA GTPase Modulates LAM Cell Migration

Small GTPase RhoA has a well-established role in regulating cell migration; the constitutive activation of RhoA results in cell transformation and metastasis (33). Importantly, RhoA activity is regulated by TSC1, which forms a cytosolic complex with TSC2 (11). Because stress fiber formation correlates with RhoA activation and its downstream effector RhoA kinase (ROCK), we examined whether inhibition of RhoA or ROCK will modulate the actin cytoskeleton and LAM cell migration. Tat-C3 toxin (a cell-penetrating form of *Clostridium botulinum* exoenzyme, which specifically catalyses the inactivation of RhoA [34]) and Y27632 (a specific inhibitor of ROCK [35]) were used to inhibit RhoA and ROCK activity. Both Tat-C3 and Y27632 abolished RhoA-dependent stress fiber formation (Figure 5A), which correlates with the inhibition of LAM cell migration (Figures 5B and 5C). In contrast, rapamycin (200 nM), a specific inhibitor of the mTOR/S6K1 signaling pathway, had little effect on LAM cell migration (Figure 5B). Importantly, this concentration of rapamycin is sufficient to abrogate LAM cell proliferation (20). To further examine the involvement of RhoA in LAM cell migration, we treated LAM cells with ROCK inhibitors Y27632 and HA-1077 at the range of concentrations that inhibit ROCK activity in other cell types (36). As seen in Figure 5C, Y27632 and HA-1077 significantly inhibited LAM cell migration in a concentration-dependent manner. In contrast, the same concentrations of Y27632 and HA-1077 had little effect on the migration of HBFs (data not shown). These data demonstrate that RhoA and ROCK activation is critical for LAM cell migration.

TSC2 and siRNA TSC1 Inhibit the Constitutive Activation of RhoA in LAM Cells

Because Tat-C3 inhibited LAM cell migration, we examined RhoA activity in LAM cells. As seen in Figure 6A, RhoA is constitutively activated in the absence of any stimuli in LAM cells; in contrast, HBFs and ASM cells showed a significantly lower level of basal RhoA activity.

Next, we examined whether TSC2 expression would affect RhoA activation in LAM cells. As seen in Figure 6B, expression of TSC2 significantly inhibited RhoA activity compared with cells transfected with control plasmid. To investigate whether

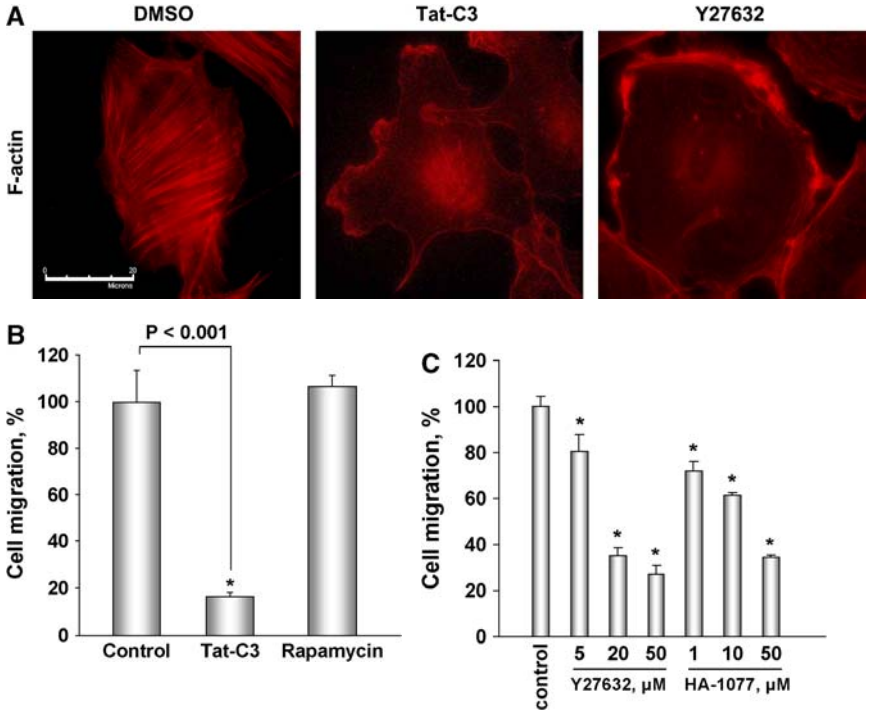

Figure 5. RhoA modulates LAM cell invasiveness. (*A*) Tat-C3 and Y27632 promote stress fiber disassembly. Serum-deprived cells were treated with either 0.5 μM Tat-C3, 20 μM Y27632, or DMSO as a control for 12 h. Stress fiber formation was detected by rhodamine phalloidin staining of F-actin. Images are representative of three separate experiments. *Scale bar* is 20 μm. (*B*) Tat-C3, but not rapamycin, inhibits LAM cell migration. Serum-deprived cells were treated with either 0.5 μM Tat-C3 or 200 nM rapamycin; the migration assay was then performed using a Boyden chamber. (C) Y27632 and HA-1077 inhibit LAM cell migration. Serum-deprived cells were treated with either Y27632 or HA-1077, followed by the migration assay. Data are representative of six replicates for each condition in two independent experiments. *$P < 0.001$ for Y27632- or HA-1077–treated cells versus control by ANOVA (Bonferroni-Dunn).

TSC1 is also required for TSC2-dependent RhoA inhibition, we used siRNA directed against TSC1 and downregulated TSC1 levels as we described previously (15). As seen in Figure 6B, downregulation of TSC1 levels resulted in significant inhibition of the constitutive RhoA activation in LAM cells. Because TSC2 inhibits RhoA activity and siRNA TSC1 is sufficient for this inhibition, we conclude that TSC1 is required for the TSC2-dependent regulation of RhoA activity. These data and our published study (15) show that TSC2 acts upstream of RhoA, and suggest that TSC2-dependent inhibition of LAM cell migration occurs due to the TSC2-dependent inhibition of RhoA activity.

TSC2 Modulates Stress Fiber Formation in LAM Cells

Because RhoA activation correlates with stress fiber formation, we next performed F-actin staining on LAM cells to detect stress fibers in LAM cells. We found that LAM cells had significantly increased stress fiber formation; the quantity of F-actin per cell in LAM cells was 44.3 ± 2.3% and 58.7 ± 1.9% higher compared with that in ASM cells and HBFs, respectively (Figure 7A). We next examined whether expression of TSC2 affects stress fiber formation in LAM cells. As shown in Figure 7B, expression of TSC2 leads to stress fiber disassembly compared with control GFP–expressed cells. Quantitative analysis of F-actin staining revealed that expression of TSC2 markedly reduced the quantity of stress fibers per cell by 27.9 ± 15.4% compared with control GFP (Figure 7D). Statistical analysis demonstrated that 98.2 ± 5.1% of GFP-transfected cells had stress fibers; in contrast, only 45.3 ± 4.2% cells transfected with TSC2 showed stress fibers (Figure 7C). These data demonstrate that TSC2 promotes stress fiber disassembly, and correlates with the inhibitory effect of TSC2 expression on RhoA activity.

DISCUSSION

The tumor suppressor proteins TSC1 and TSC2 have well-established roles in regulating cell growth, and loss of their functions is associated with LAM disease. Little, however, is known about the role of TSC1 and TSC2 in neoplastic LAM cell dissemination. This study shows that TSC2 plays a critical role in regulating LAM cell migration and invasiveness. Primary cultures of LAM cells show increased migratory activity and invasiveness. These effects are abolished by TSC2 re-expression. Importantly, TSC2-dependent regulation of LAM cell migration involves the modulation of RhoA activity, inhibition of which results in the abrogation of LAM cell migration. We also found that TSC1 is required for the regulation of RhoA activity in LAM cells. Collectively, these data suggest that loss of TSC2 may promote cell migration and invasiveness by specifically upregulating RhoA activity, which, in turn, requires TSC1.

Although the pathobiology of LAM is generally linked to abnormal cell growth, the neoplastic nature of LAM has been suggested by recent clinical and genetic evidence (16). Until recently LAM and TSC tumors were considered benign. Compelling evidence, however, supports a neoplastic model for LAM (17–19), suggesting a link between loss of TSC2 function and cell invasion and metastasis. Thus, the naturally occurring *TSC2* mutations and loss of heterozygosity (LOH) for *TSC2* in the Eker rat leads to renal adenoma and carcinoma, some of which become malignant and metastasize to the lung, pancreas, and liver (37). The hematogenous lung metastases of malignant uterine leiomyosarcoma were also identified in the Eker rat (38). Established TSC2-deficient ELT3 smooth muscle cells derived from Eker rat uterine leiomyomas develop tumors in nude mice (39). TSC1−/− and TSC2−/− embryonic fibroblasts showed anchorage-independent growth and colony formation in soft agar, indicating that loss of TSC2 induces growth independent

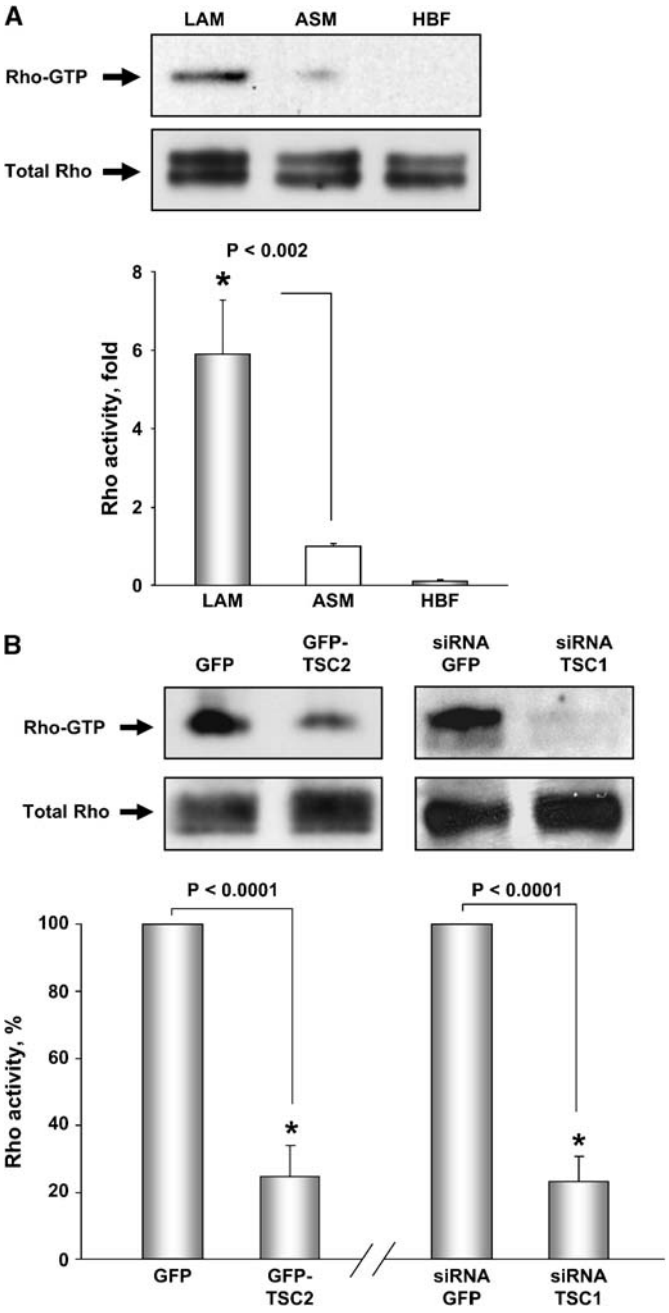

Figure 6. (*A*) Rho activity is increased in LAM cells. Serum-deprived LAM cells, ASM cells, and HBFs were subjected to the Rho activation assay. *Top panel* represents Rho-GTP pulled down with Rhotekin-RBD agarose and total Rho; images are representative from three independent experiments. *Bottom panel* shows the statistical analysis of basal Rho activity in LAM cells, ASM cells, and HBFs. Data represent mean values ± SE from three independent experiments by ANOVA (Bonferroni-Dunn). (*B*) TSC2 and siRNA TSC1 inhibit Rho activity in LAM cells. Cells were transfected with either GFP-TSC2 and control GFP, or siRNA TSC1 and siGLO RISC-Free siRNA control, followed by the Rho activity assay. *Top panel* represents Rho-GTP pulled down with Rhotekin-RBD agarose and total Rho detected with anti-Rho antibodies. *Bottom panel* shows quantitative analysis of three independent experiments performed using the Gel-Pro Analyzer Software. *$P < 0.001$ for GFP-TSC2 versus control GFP; siRNA TSC1 versus control siRNA by ANOVA (Bonferroni-Dunn).

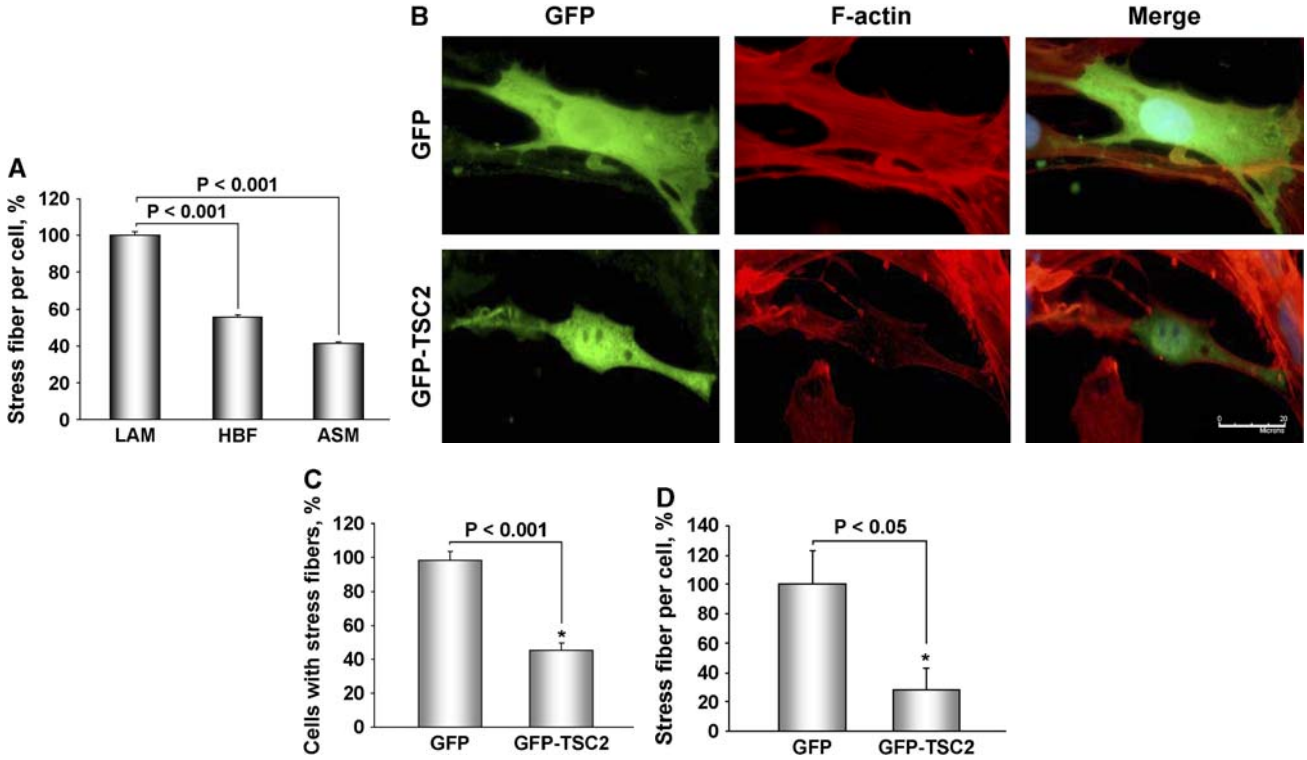

Figure 7. TSC2 modulates stress fiber formation in LAM cells. (*A*) LAM cells have increased stress fiber formation. Serum-deprived LAM cells, HBFs, and ASM cells were subjected to staining with rhodamine-phalloidin to detect stress fibers. Quantitative analysis of F-actin staining was performed using a Nikon Eclipse TE2000-E Microscope and the Gel-Pro Analyzer Software. F-actin optical density of LAM cells was taken as 100%. Data represent mean values ± SE from three separate experiments by ANOVA (Bonferroni-Dunn). (*B*) TSC2 expression promotes stress fiber disassembly. Cells were transiently transfected with control GFP or GFP-TSC2 plasmids; cells were then serum deprived and were stained with rhodamine-phalloidin to detect F-actin (*red*) and immunostained with anti-GFP antibody to detect transfected cells (*green*). Images are representative of three separate experiments. (*C*) Statistical analysis of stress fiber formation in LAM cells transfected with TSC2. F-actin staining was quantified by visual analysis of digital images taken using a Nikon Eclipse TE2000-E microscope. Data represent the percentage of cells with stress fibers per total number of cells expressing GFP or GFP-TSC2 taken as 100%. Data represent mean values ± SE from three separate experiments by ANOVA (Bonferroni-Dunn). (*D*) Quantitative analysis of stress fiber disassembly induced by TSC2 expression. The stress fiber formation in cells expressing GFP or GFP-TSC2 was assessed by analyses of F-actin optical density using the Gel-Pro Analyzer Software. F-actin optical density of cells transfected with GFP was taken as 100%. Data represent mean values ± SE from three separate experiments by ANOVA (Bonferroni-Dunn).

of adhesion, which is one of the characteristics of the invasive cell phenotype (40, 41). Metastatic tumors in lung were found in TSC1+/− mice with renal carcinoma (42). We demonstrate that primary cultures of human LAM cells have increased migratory activity and invasiveness that is abrogated by TSC2 re-expression, which indicates that loss of TSC2 function promotes invasive, neoplastic cell migration associated with LAM.

Little is known about the involvement of TSC2 in the regulation of cell motility. TSC2 forms a cytosolic heterodimer with TSC1 encoded by the tumor suppressor gene *TSC1*. Erk-dependent phosphorylation of TSC2 leads to dissociation of the TSC1/TSC2 complex, and promotes oncogenic transformation (43). It is shown that TSC2 binds TSC1 through its TSC1-binding domain, which overlaps with the Rho-activating domain of TSC1 (11), suggesting that TSC1/TSC2 complex formation may be involved in the regulation of RhoA GTPase activity and, ultimately, cell migration. Dysregulation of TSC2 function due to TSC2 loss or mutation may result in dysregulation of TSC1/TSC2 complex formation, upregulation of RhoA GTPase, and, finally, enhanced cell motility. Our study shows that unstimulated LAM cells show increased RhoA activity, and inhibition of either RhoA GTPase or its downstream effector ROCK abrogates the increased migratory activity of LAM cells. These data correlate with studies demonstrating that RhoA activity is necessary for increased migration of human tumor cells (33). Further, upregulation of RhoA GTPase as well as RhoA effector protein ROCK are commonly observed in human cancers and are often associated with more invasive and metastatic phenotypes (33). Importantly, our data demonstrate that both TSC2 re-expression and down-regulation of TSC1 levels with specific siRNA abrogate abnormal RhoA activity in LAM cells. Collectively, our data suggest that loss of TSC2 function leads to TSC1-dependent RhoA activation, potentially through the dysregulation of the TSC1/TSC2 complex, which, in turn, promotes LAM cell migration and invasiveness. Our finding that expression of TSC2 inhibits RhoA activity in LAM cells contradicts a previous paper by Astrinidis and coworkers which demonstrated that re-expression of TSC2 in TSC2-deficient ELT3 cells results in RhoA activation and inhibition of cell migration (44). These differences in experimental data may occur due to differences in experimental approaches. We used *transient* TSC2 expression, which promotes the "classical" lamellopodial or mesenchymal motility driven by Rac1 activation and inhibition of RhoA. Astrinidis and colleagues used ELT3 and MDCK clones with *stable* overexpression of TSC2. Because RhoA activation is required to promote a rounded bleb-associated mode of motility in some tumor cell

lines, it is possible that stable overexpression of TSC2 had changed the cell phenotype, which requires activation of RhoA (33).

TSC1/TSC2 regulates cell growth and proliferation through the mTOR/S6K1 signaling pathway, which is abrogated by rapamycin, thus inhibiting mTOR. In TSC2-deficient cells, actin remodeling is insensitive to rapamycin, suggesting that the rapamycin-sensitive mTOR pathway does not contribute to biochemical events that occur as a part of the regulatory mechanisms of cell dynamics (15). The role of TOR in regulating the actin cytoskeleton has been well known in yeast (45); in mammalians, however, this function of mTOR has remained an enigma. Recently, it was shown that mTOR, as a part of the rapamycin-insensitive mTOR complex 2 (mTORC2), regulates the actin cytoskeleton through Rac1 and PKCα (46, 47). Our current study also shows that LAM cell migration is rapamycin-independent. Further studies are needed to establish the relationship between the TSC1/TSC2-dependent regulation of the actin cytoskeleton, cell adhesion and motility, and mTORC2, and their relative contribution to LAM pathobiology.

Until recently TSC1 and TSC2 were generally considered to be regulators of protein translation and cell growth, and loss of function mutations of *TSC1* and *TSC2* promote abnormal cell growth associated with LAM pathology. However, loss of only TSC2 is associated with disease severity (5). Our study identifies that TSC2 functions not only as a negative regulator of cell growth, but also as critical regulator of actin remodeling, cell migration, and invasiveness. Thus, loss of TSC2 function may not only results in abnormal cell growth, but also in increased cell migration and invasiveness, which may lead to LAM disease severity. Our data further suggest that tumors characterized by TSC2 dysfunction may be amenable to therapies that abrogate activated RhoA. Further studies are necessary to determine the mechanism(s) by which TSC2 suppresses RhoA activity, cell migration, and invasiveness. Although LAM is a rare interstitial lung disease, in terms of research, its importance cannot be overstated because these studies specifically improve our understanding of the molecular signaling pathways regulating mesenchymal cell migration, such as vascular or airway smooth muscle cell or interstitial lung fibroblast migration (48); this is critically important in diseases such as pulmonary arterial hypertension, asthma, idiopathic pulmonary fibrosis, and interstitial lung disease.

***Conflict of Interest Statement*:** None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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